JAGGER and *AGP7*, putative SEEDSTICK targets involved in pollen tube attraction

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo

A escassez de comida prevista para as próximas décadas é o problema mais urgente que a humanidade enfrenta atualmente. Para ultrapassar este desafio de forma sustentável, devemos concentrar os nossos esforços em obter maior rendimento da terra arável já em uso. De forma a melhorar o rendimento das colheitas, precisamos em primeiro lugar de adquirir um conhecimento profundo sobre o processo de reprodução sexual em angiospérmicas, que representam a maioria das colheitas, e cujo propósito final é a produção de semente por dupla fecundação.

O projeto SEXSEED tem como objetivo estudar o fator de transcrição SEEDSTICK, um regulador mestre da produção de semente, e a rede através da qual funciona. Dados obtidos através de ChIP-Seq apontaram *JAGGER* e *AGP7* como possíveis interatores de SEEDSTICK, e como tal peças nesta rede. Estas são ambas proteínas arabinogalactânicas, com uma âncora GPI prevista. Estudos recentes com a proteína LORELEI, também envolvida no processo reprodutivo, que possui uma âncora GPI, demonstraram que esta é essencial para a localização subcelular da proteína, mas não para a sua função. JAGGER tem já uma função conhecida na atração do tubo polínico, estando envolvido na cadeia sinalizadora que leva à degeneração da sinergídea persistente. No entanto, estudos anteriores sugerem o envolvimento de outra molécula, que desempenhe o mesmo papel na cadeia sinalizadora. Com base nestas observações, propusemos a seguinte hipótese de trabalho: AGP7 funciona de forma redundante com JAGGER, e ambas dependem da localização subcelular, determinada pela âncora GPI, para desempenhar os seus papeis.

No decorrer deste trabalho conduzimos analises de fenótipo num mutante *agp7* homozigótico, procuramos obter um duplo mutante *jagger/agp7*, e preparamos fusões com proteínas repórter. Estas proteínas de fusão vão permitir nos determinar, não só a localização da proteína em células e tecidos, mas também verificar se são capazes de conseguir o resgate do fenótipo mutante. De forma a estudar o envolvimento dos domínios do sinal GPI na localização e função de JAGGER, preparamos construtos deletérios.

Estas ferramentas serão uteis para estudos futuros, ajudarão a esclarecer os mecanismos da acora GPI no que diz respeito à dinâmica de proteínas, e o nosso entendimento geral acerca do papel das AGPs na reprodução sexual – desta forma contribuindo para que sejam construídos alicerces essenciais, sobre os quais a biotecnologia pode construir para o melhoramento futuro de produção de colheitas.

Abstract

The announced food scarcity in the decades ahead is the single most pressing problem humankind faces today. To sustainably overcome this challenge, we must turn our attention to achieving higher yields from the arable land already in use. To improve crop yields we need to attain a deep fundamental knowledge of the sexual reproduction process in angiosperms, who represent most of agricultural crops, and whose ultimate goal is the production of seed by double fertilization.

This study is part of the SEXSEED consortium, who came together to be a part of this solution. SEXSEEDs project aims to study SEEDSTICK, a master regulator of seed production, and the intricate network in which it works. CHiP-Seq data highlighted JAGGER and AGP7 as putative SEEDSTICK targets, and thus players in this network. Both these proteins are arabinogalactan proteins with a predicted GPI-anchor. Recent studies with the GPI-anchored protein LORELEI, also involved in angiosperm double fertilization, demonstrated that the GPI anchor is essential for protein subcellular localization but not for its function. JAGGER is known to be involved in pollen tube reception by playing a central role in the persistent synergid degeneration, however, the results obtained in previous studies have hinted that another signalling molecule, yet undiscovered, is involved in the same signalling role.

Based on previous observations comparing AGP7 and JAGGER, we postulated the working hypothesis that it is AGP7 which works redundantly with JAGGER, and that both proteins depend on the subcellular localization, determined by the GPI anchor, to perform their roles. In the course of this work we performed phenotype analyses on an *agp7* homozygous mutant, we worked on achieving a double homozygous jagger/agp7 mutant, and we prepared fusions with reporter proteins. These fusion proteins will allow us to determine not only tissue and subcellular localization, but also mutant phenotype rescue in *jagger* mutant plants. To study the involvement of the GPI signal domains (present in the nascent protein) in JAGGERs localization and function, we prepared deleterious constructs with GPI signal mutations.

These tools will be of value in future studies and help to shed light into the GPI anchor mechanism in respect to protein dynamics, and to our understanding of AGPs in sexual reproduction, thus contribute in laying essential foundations on which biotechnology can build upon for the future improvement of seed production.

Keywords: Arabinogalactan Proteins; Pollen tube attraction; GPI signal; Double fertilization.

Table of Contents

Index - Figures

Index - Tables

Abbreviations and Acronyms

A.thaliana, At - Arabidopsis thaliana

A.tumefaciens - Agrobacterium tumefaciens

AGP - Arabinogalactan Protein

AMOR - Activation Molecule for Response capability

bp - base pair

ChIP - Chromatin Imunoprecipitation

CRP - Cysteine Rich Polypeptides

cYFP - citrine Yellow Fluorescent Protein

DEFL - Defensin-Like

DNA - Deoxyribonucleic acid

E.coli - Escherichia coli

ER - Endoplasmic Reticulum

FAO - Food and Agriculture Organization

FER - FERONIA

GAP - GPI Anchored Protein

Gly - glycine

GPI - glycosylphosphatidylinositol

HM - Homozygous

Hyp - hydroxyproline

HZ - Heterozygous

LB - Luria Bertani medium

LP - Left Primer

LRE - LORELEI

Min - minute

mL – milliliter

mM - millimolar

MS - Murashige & Skoog

nm – nanometer

N.benthamiana, Nb – Nicotiana benthamiana

OD – Optical Density

ON - Overnight

PCR - Polymerase Chain Reaction

PLC – Phospholipase C

Pro - proline

RP - Right Primer

RT - Room Temperature

STK - SEEDSTICK

TTS - Transmitting Tract Specific

UTR - Untranslated Region

UV - ultra violet radiation

WT - Wild-Type

µg - microgram

µL - microliter

ω-site - omega site

1. Introduction

1.1. Scientific Context of the Thesis

By the year 2050 two billion more people are predicted to inhabit the Earth. This leaves us face to face with two major challenges we need to overcome in the immediate future: feeding the ever-growing population, while at the same time reducing the agricultural footprint, to allow the regeneration of the planet resources at a sustainable rate. Until now we have "created" arable land by cutting down forests and ploughing grasslands, at much too high a cost: damaging ecosystems and biodiversity in some cases to a point of no return. We can no longer afford to go down this path. It is crucial that we now turn our attention to boosting yields in the already existent farmlands.

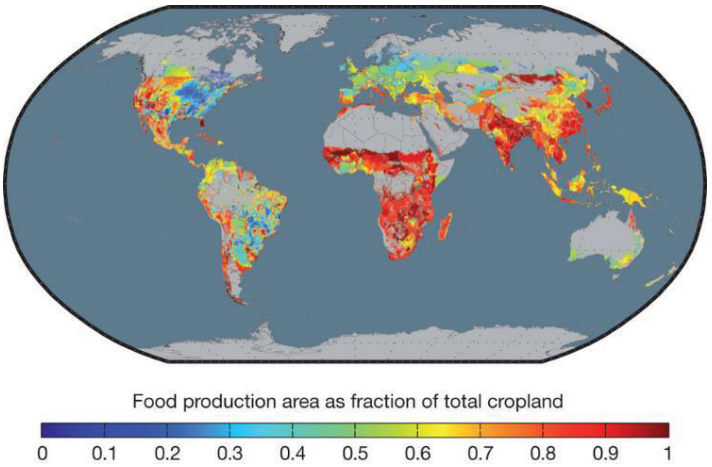

Figure 1 - Representation of the cropland area allocation to different uses in 2000. Colour grading compares varying degrees of food production efficiency. Adapted from Foley et al., 2011

Figure 1 represents the world's total cropland that is dedicated either to growing food crops directly and indirectly (through animal feed), or to bioenergy crops, seed, and other industrial products (a predicted 3%), highlighting the striking disparities between crop yields around the globe. Based on their analyses, Foley and collaborators (2011) proposed a five-step solution to overcome these challenges:

1) Shifting diets away from meat

2) Reducing food waste

3) Stopping the expansion of agriculture's footprint

4) Closing the world's yield gaps

5) Using resources more efficiently

Cutting meat would allow us to redirect the produce spent on livestock directly to human sustenance, and to prevent creating additional living space and pasture for livestock, in time possibly allowing us even to retrieve land already in use for these purposes.

Reducing food waste would also increase food availability, if we take into account that a FAO study [Gustavsson *et al.*, 2011] predicted that one-third of food is never consumed due to being discarded, degraded or attacked by pests along the supply chain. Both these changes are dependent on a mentality change by the consumer, and the main role of science here is to supply useful data, so that the general public may make more adequate and informed choices.

However, stopping the agricultural footprint, closing the worlds yield gaps and using resources efficiently are a scientific problem in their core, and the solution starts by achieving crop plants with significantly higher yields per area unit: being able to withdraw more produce from each single plant will reduce the amount of land and water needed as a whole, to name only the obvious advantages. To take part in this solution, the SexSeed (Sexual Plant Reproduction - Seed Formation) consortium was created, in which this project is integrated.

1.2. SexSeed Consortium

The purpose of SexSeed is to study a master regulator of seed production, SEEDSTICK, and the intricate network in which it works. SEEDSTICK (STK) is a transcription factor belonging to the MADS-box family, which is defined by the presence of a conserved motif that encodes a DNA binding MADS domain. This transcription factor is one of the master regulators of the three decisive events that determine viable seed formation: ovule development, double fertilization, and seed/fruit development [Baker et al., 1997; Mizzotti et al., 2012].

ChIP-sequencing is a technique that combines chromatin immunoprecipitation with massive DNA sequencing, allowing us to determine protein/DNA interactions. Preliminary data from STK ChIP-sequencing identified JAGGER and AGP7 as putative STK targets [Mizzotti, unpublished data].

1.3. Sexual Reproduction in Angiosperms

To improve crop yields we need, first and foremost, to achieve a thorough knowledge of the sexual reproduction process in angiosperms, which represent most of agricultural crops [Kesseler and Stuppy, 2006]

The flowering plants, angiosperms, are seed-producing plants whose lifecycle alternates between two phases, sporophytic and gametophytic. The sporophytic generation is diploid and constitutes the adult plant, which is able to generate two distinct kinds of spores – microspores and megaspores – that will give rise to the male and female gametophytes, respectively. The gametophytic generation is haploid and its main function consists in the formation of male and female gametes. The lifecycle is completed when both gametes unite to form a zygote which will develop into the sporophyte. It is a characteristic, defining feature of angiosperms to reproduce by double fertilization, the fusion of two distint sperm cells (male gametes) with the two female gametes, the egg cell and central cell. [Yadegari and Drews, 2004]

1.3.1. The Male Gametophyte

The male gametophyte in angiosperms is a highly specialized structure, the pollen grain. It arises from a diploid pollen mother cell who undergoes meiotic division to produce a tetrad of haploid microspores. These microspores are released and submitted to an asymmetric division that produces a bicellular pollen grain, with a small generative cell encased within the cytoplasm of a large vegetative cell. This generative cell will go through a further mitotic division, that will generate the two twin sperm cells (the haploid male gametes). This development of the pollen grain is depicted in figure 2.

The pollen grain has two main challenges to overcome in order to fulfil its goal: it must find its way to a receptive pistil, and then successfully deliver both sperm cells to the embryo sac (the female gametophyte) to achieve double fertilization. The first can be attained by simply dehiscing onto the pistil or by being transported by insects or wind. To accomplish the second, the pollen grain must complete a journey that starts with a pollen tube germinating from the pollen grain and growing trough the pistil tissues until reaching its final destination, the ovule. [McCormick, 1993; Borg and Twell, 2010; Palanivelu and Tsukamoto, 2012]

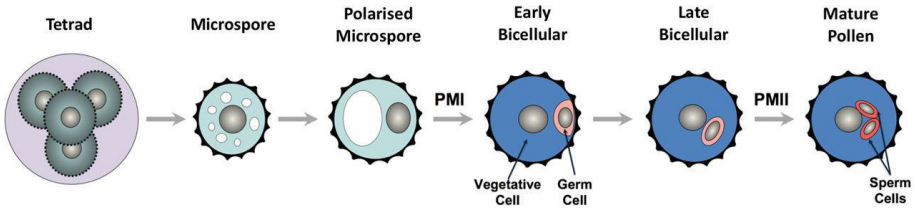

Figure 2 - Male Gametophyte Development in *Arabidopsis*. Adapted from Borg and Twell, 2010

1.3.2. The Female Gametophyte

In angiosperms, the female gametophyte or embryo sac is nested inside the protective layers of the ovule. Its development begins when the megaspore mother cell undergoes meiosis. From the four resulting cells, only one survives and undergoes three mitotic divisions, resulting in a female gametophyte containing eight nuclei. These eight nuclei distribute themselves in a spatially-specific order, differentiating in three antipodal cells, two synergid cells, one egg cell and a central cell with two polar nuclei, as depicted in figure 3.

In *Arabidopsis thaliana,* prior to the pollen tube arrival the three antipodal cells degenerate and the two polar nuclei fuse to form a homodiploid central cell. The synergid cells contain an elaborate, thickened cell wall at their micropylar poles, the filiform apparatus, that appears to be involved in pollen tube guidance, as well as cytoskeletal elements that are likely involved in migration of the sperm cells towards their fertilization targets. [Punwani and Drews, 2008; Sprunck & Groß-Hardt, 2011; Palanivelu and Tsukamoto, 2012]

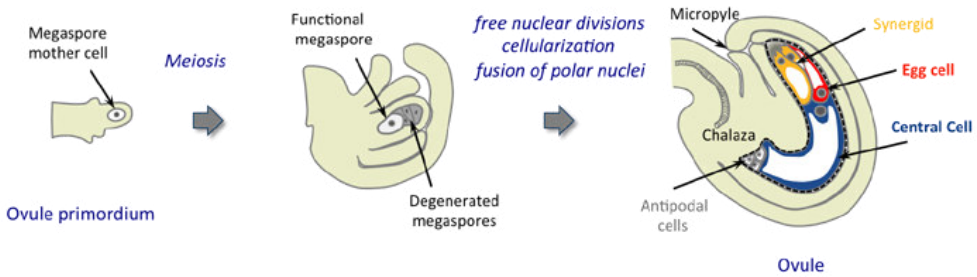

Figure 3 – Female Gametophyte Development in *Arabidopsis*. Adapted from Sprunck & Groß-Hardt, 2011

1.3.3. Polen-Pistil Interactions

From the moment a pollen grain reaches the pistil until the male gametes are delivered to the embryo sac, the tip-growing pollen tube interacts with several distinct pistil cell-types: stigma, style, transmitting tract, septum, funiculus, integument, and synergid cell. Figure 4 illustrates a schematic representation of the pollen tube growth and guidance.

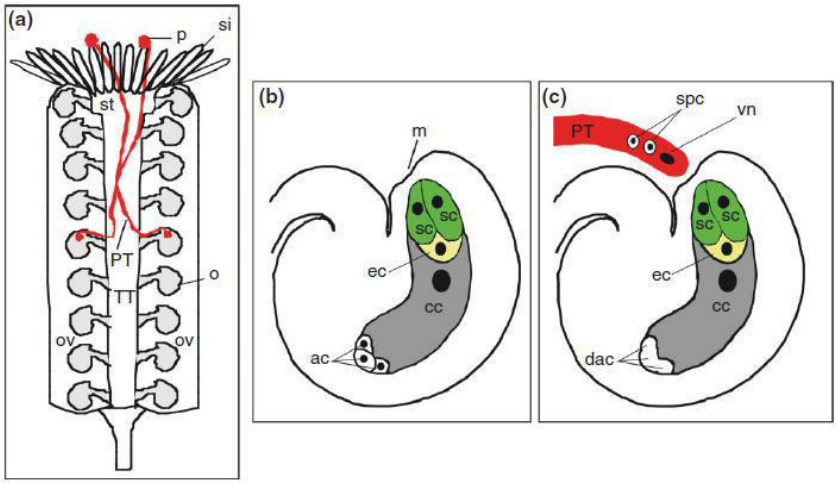

Figure 4 - Pollen tube growth and guidance to ovule micropyle. (a) Pollen tube growth within an *Arabidopsis* pistil. Pollen grains (p) on the stigma (si) germinate and extend pollen tubes (PT, red) through the style (st) and transmitting tract (TT) before entering one of the two ovary (ov) chambers to target an ovule (o). (b) female gametophyte within an ovule. (m) micropyle; (ac) antipodal cells. (c) Pollen tube carrying sperm cells and vegetative nucleous approaching the micropyle. Adapted from Palanivelu and Tsukamoto, 2012

Compatible pollen recognition by a receptive stigma is the first step in this chain: adhesion to the stigma is a selective process where compatible pollen grains bind with high affinity to the stigma, while incompatible pollen fails to adhere. After hydrating, the pollen grain is physiologically activated in order to germinate: a protruding tube begins to grow. In *Arabidopsis*, the pollen germination is known to occur in less than 30 minutes. [Zinkl *et al.*, 1999; Palanivelu and Tsukamoto, 2012]

Pollen tube guidance along the tissues takes places as a long distance guided polar cell growth, and can be divided in two consecutive phases: a sporophytic phase in which the pollen tube grows within the transmitting tract and a gametophytic phase in which it grows along the funiculus and enters the micropylar opening of the ovule. [Chen, *et al.*2007]

Sporophytic phase

It has been demonstrated that style tissues serve not only as conduit for the pollen tube to reach the transmitting tissue and the ovule, but they also enable competence of the pollen tube to perceive the guiding cues provided from the female tissues [Yadegari and Drews, 2004]. Studies conducted in *Torenia fournieri* discovered the diffusible factor AMOR (Activation MOlecule for Response capability) and verified it originated from the sporophytic tissue of the ovule, as laser ablation assays showed that the embryo sac, even the synergid cells, were not required – for even when the entire embryo sac was compromised the AMOR molecules where still detected. This ovular factor is critical to induce competency on pollen tubes, that is, to make them capable of responding to the attraction signal from the synergid cell, as represented in figure 5. [Mizukami *et al.*, 2016]

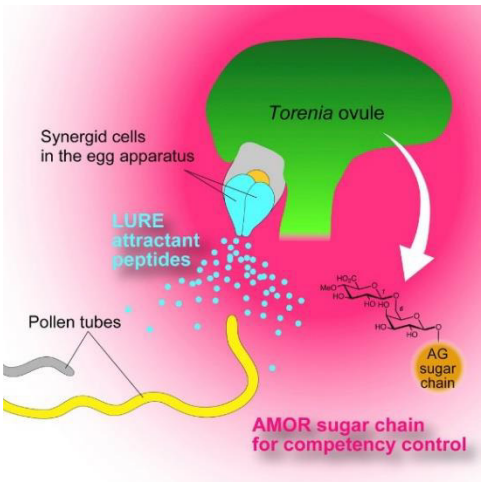

Figure 5 - Cartoon representation of AMOR molecule pollen tube competency control in *Torenia fournieri*. Reproduced from Mizukami et al., 2016.

On the transmitting tissue, it has been demonstrated that calcium helps to control the direction of the pollen tube by regulating the actin cytoskeleton disposition. Arabinogalactan proteins (AGPs) have also been identified as important molecules along this path, performing several signaling functions: acting as receptors of extracellular signals and interacting with transmembrane proteins, ultimately affecting the calcium channels; acting as potential attractants that direct growth, among others. The extracellular matrix alone presents several AGPs to the travelling pollen tube: in *Nicotiana*, Transmitting Tract Specific (TTS) and a 120kDa glycoprotein (120K) are AGPs found to stimulate the pollen tube growth, mediate in vitro attraction, and self-recognition. [Scott and Stead (eds.), 1994; Taylor and Hepler, 1997; Higashyama and Takeushi, 2015]

Gametophytic phase

As the pollen tube reaches the embryo sac surroundings, the synergid cells produce specific molecules, released by the filiform apparatus, attracting the pollen tube to grow around this apparatus and penetrate the receptive synergid cell. Okuda and collaborators (2009) identified in *Torenia fourieri* two Cysteine Rich Polypeptides (CRP) originating in synergid cells that constitute a diffusible, species-specific attractant signal for pollen tubes, who they called LUREs. Takeuchi and Higashiyama (2012) identified a similar cluster of Defensin-like (DEFL) genes in *A. thaliana*, designated the AtLURE1 genes, that encode pollen tube attractants. Defensins are antimicrobial peptides that play a role in innate immunity in eukaryotes. Plant DEFL peptides play a role not only in this innate immunity system, but they are also involved in male–female interactions in plant sexual reproduction. The authors demonstrated that AtLURE1 peptides are produced by the synergid cells and diffused to the funicular surface through the micropyle, and that when these peptides are downregulated, micropylar guidance of the pollen tube to the synergid cell is impaired.

The pollen tube is thus guided to enter the micropyle and arrive at the receptive synergid cell. Synergid cells are positioned surrounding the egg cell, and possess a specialized structure, the filiform apparatus, that consists of thick cell wall projecting several finger-like invaginations into the synergid cytoplasm. The pollen tube grows along the filiform apparatus, enters the synergid cell, and arrests its growth. Immediately the two sperm cells are burst-released into the embryo sac, one of which fuses with the egg cell to form an embryo, and the other fuses with the central cell to form the endosperm – this process in which the two male gametes fuse with the two female gametes is named double fertilization, a unique feature of angiosperms, and poses as hallmark between the diploid and haploid generations. [Palanivelu and Tsukamoto, 2012; Pereira, *et al.*, 2015]

As soon as double fertilization is achieved, the persistent synergid degenerates and ceases to release attractants: this ensures one-on-one pairings of male and female gametes to prevent polyspermy, which would likely lead to reproductive failure. It is the fusion of the persistent synergid cell with the expanding endosperm that inactivates it [Maruyama et al., 2015]. If each step of this chain is successful (fig. 6), a seed is born.

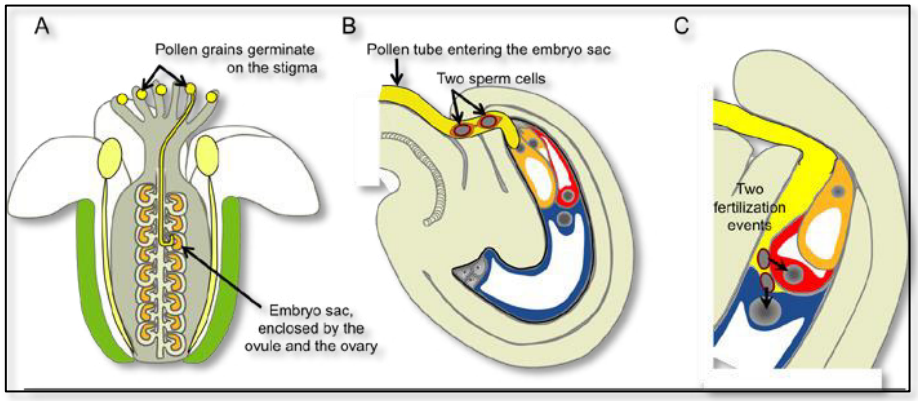

Figure 6 - Schematic representation of the pollen tube journey through an *Arabidopsis* pistil. A- Pollen germination and tube growth; B – Pollen tube entrance in the embryo sac; C- double fertilization. Adapted from Sprunk (2010)

1.4. The Arabinogalactan Protein Family

What exactly is an arabinogalactan protein? This has proven to be a difficult definition to pin down, due to the diversity found in its members. The AGP family is one of the most complex macromolecule families found in plants, and more than one AGP category as been defined. Here we will focus on the definition of classical AGPs, represented in figure 7. The protein backbone in classical AGPs, is mainly composed of repetitive proline-rich dipeptide motifs, and typically accompanied by serine, threonine, or alanine. [Showalter and Basu, 2016]

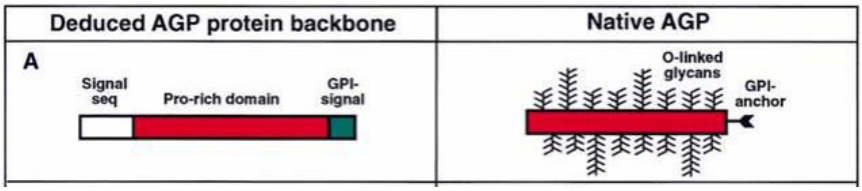

Figure 7 - Schematic representation of a classical AGP. Deduced from DNA sequence (left-hand panel), and the predicted structures of the native AGP after processing and post-translational modification (right-hand panel). Adapted from Gaspar et al.,2001

This nascent protein will go through several post translational modifications, namely the hydroxylation of several prolines by the enzyme prolyl hydroxylase, in the ER. This enzyme will add an hydroxyl group to the amino group in proline – turning it into hydroxyproline (hyp). It is so far unclear what criteria determines which prolines are hydroxylated and which aren't.

Experimental observation so far shows that SP and AP motifs are always hydroxilated, while TP motifs are only seldom hydroxylated [Showalter, 2001].

This modification is a vital step for the second post translational modification, which is glycosylation by glycosyltranferases, who attach complex carbohydrates (that consist mainly, as the AGP name implies, of galactan and arabinose) to hyp residues. The pattern to which the glycans moieties are added is still cause for debate, but the hyp contiguity hypothesis is widely accepted: this hypothesis states that non-continuous Hyp residues will receive an AG polyssacharide while continuous hyp residues will receive a short arabino-oligossacharide. This type of O-glycosylation is only present in plants and green algae. [Shpak *et al.*, 2001; Mohnen and Tierney, 2011] At last, the protein backbone possesses in its C terminal a GPI signal that induces the addition of a glycosylphosphatidylinositol (GPI) anchor.

Additionally, classical AGPs can also be defined by a chemical property: the ability to react with a syntethic dye called Yariv reagent.

1.4.1. The GPI anchor

The GPI anchor is a post-translational modification that tethers a protein to the extracellular leaflet of the plasma membrane. This modification is conducted in proteins that possess a GPI signal, by the transamidase complex present in the lumen of the Endoplasmic Reticulum (ER), [Ellis et al, 2010]. Figure 8 illustrates the GPI signal, which contains an omega region, composed of 3 aliphatic amino acids with short side chains. The first of these amino acids is the omega site (ω-site), and constitutes the binding site for the anchor itself [Schultz et al.1998; Eisenhaber et al., 2003]

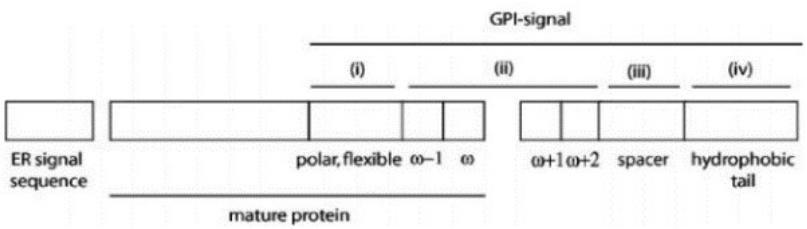

Figure 8: The C-terminal GPI lipid anchor signal. The scheme illustrates the two signals that are necessary for GPI lipid anchoring: the N-terminal ER export signal and the C-terminal transamidase recognition signal. Adapted from Eisenhaber et al., 2003.

The proposed mechanism for GPI anchor synthesis and attachment (as schematized in figure 9), is thought to occur along the following steps: GPI moiety is synthesized on the cytoplasmic side of the ER; at the same time, protein backbone is being inserted into the ER co-translationally; The transamidase complex brings the two together by cleaving the protein backbone on the ω-site and attaching the GPI anchor to it (this is also the time at which Pro residues are being modified to Hyp residues) [Ellis *et al.,* 2010; Cheung, 2014].

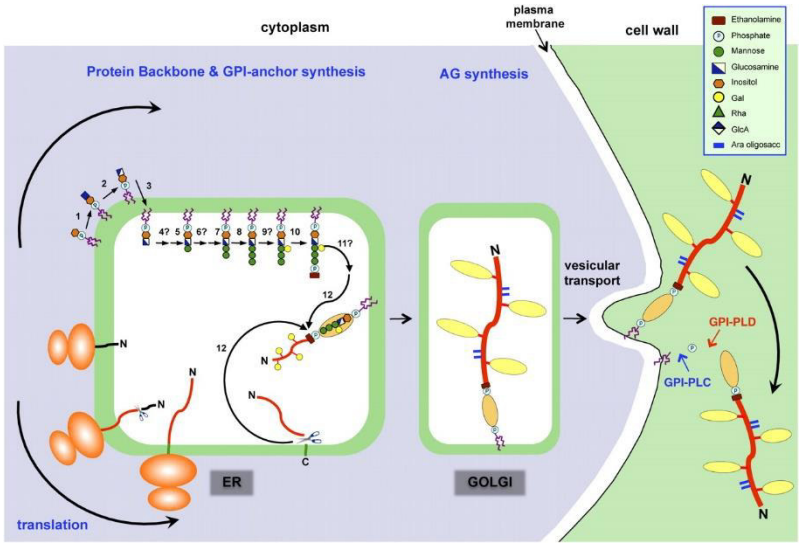

Figure 9 – GPI anchor synthesis and attachment to protein backbone simultaneously translated in the ER. Also represented is the glycan addition in the Golgi Apparatus and the vesicular transport of the completed AGP to the cell surface. Adapted from Ellis *et al*., 2010

Not much is known, so far, regarding the precise function of the GPI anchor in plants. Liu and collaborators (2016) have conducted a structure-function characterization of LORELEI (LRE), a GPI-Anchored Protein (GAP) preferentially expressed in the synergid cells. LRE plays an essential role in pollen – synergid interaction, working together with the receptor-like kinase FERONIA (FER) in pollen tube reception into the synergid cell: more specifically, in the growth arrest and subsequent burst-release of sperm cells. *lre* mutants showed abnormal growth of pollen tubes, coiling inside the synergid cell.

Through an elegant study, the authors determined that the presence of the GPI-anchor determines the protein subcellular localization, but surprisingly, the presence or absence of the GPI-anchor did not prevent the protein from fulfilling its role (although it impaired it) demonstrating that subcellular localization is helpful but not essential for LRE function (Liu *et al*., 2016).

1.4.2. JAGGER and AGP7

The role of JAGGER in pollen tube attraction to the female gametophyte has been uncovered by Pereira and colleagues (2016): JAGGER, the AGP4, is a key player in preventing the arrival of multiple pollen tubes to a successfully fertilized ovule (in *Arabidopsis thaliana*), a phenomenon denominated "polytubey" phenotype. It is involved in the signalling pathway that leads to persistent synergid degeneration by fusion with the expanding endosperm. When successful double fertilization occurs, egg cell and central cell each send an independent signal to the persistent synergid, that triggers its degeneration. The egg cell does this by ethylene signalling, which triggers a transduction cascade in which JAGGER is thought to be involved.

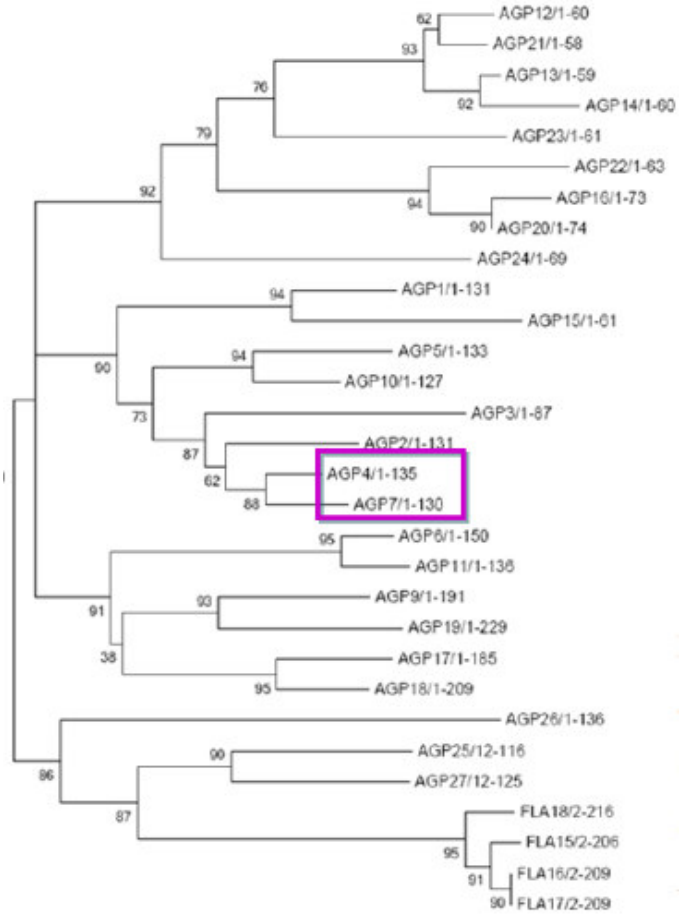

Figure 10: Phylogenetic analysis of the AGP family in *A. thaliana*. Obtained by comparison of the coding sequences of the indicated AGPs. Highlighted in purple is the similarity between AGP7 and AGP4. Adapted from Pereira et.al., 2014.

However, it was observed that *jagger* null mutant homozygous plants did not present a fully penetrant phenotype, which suggests that another player may be involved. AGP7 appears to be the most closely related in the AGP family to JAGGER, sharing a high degree of similarity between their amino acidic sequences, as shown in figure 10. This similarity might be an indicator of functional redundancy, and given the phenotype results obtained in jagger mutants, previously referred, it is proposed that AGP7 might play a redundant role with JAGGER.

1.5. Why study reproduction in *Arabidopsis*?

Although not of agronomic value, *Arabidopsis thaliana* is an invaluable plant for research purposes. Having its genome fully sequenced, its anatomy and physiology extensively studied, simple and well-established transformation procedures and a large number of mutant lines readily available, makes this small flowering plant the model organism of choice when it comes to cellular and molecular plant biology. Particularly in reproductive studies, *A. thaliana* brings the added benefits of a short life cycle, being prolific in seed production by auto pollination and easily cultivated in a restricted environment due to its relatively small size. Most importantly, *Arabidopsis* research is easily translated in knowledge to use in crop plants. [Bevan and Walsh, 2005; Koornneef and Meinke, 2009].

1.6. Objectives

It is then the purpose of this work to characterize AGP7 putative role in pollen tube attraction, prepare fusion proteins to observe JAGGER and AGP7 subcellular localisation, and analyse the ability of these fusion proteins to rescue JAGGERs mutant phenotype. We will also prepare GPI signal mutants in order to correlate the results obtained with the importance of the GPI signal domains in both the protein localization and function. The proposed work will help lay essential groundwork in sexual plant reproduction knowledge.

2. Methodology

2.1. Obtaining cYFP constructs

In order to study both AGPs subcellular localization, classical molecular biology tools were employed to obtain chimeric fluorescent proteins. To understand the importance of particular domains within the GPI signal in the nascent protein composition, mutated versions of the fluorescent fusion protein were also obtained. These constructs were used to stably transform *Arabidopsis thaliana* plants of different backgrounds, employing *Agrobacterium tumefaciens* mediated methods, with the purpose of obtaining transgenic *A. thaliana* homozygous lines.

An attempt to validate the constructs functionality through transient expression in *Nicotiana* plants was carried out. Expression will be evaluated by confocal microscopy observation.

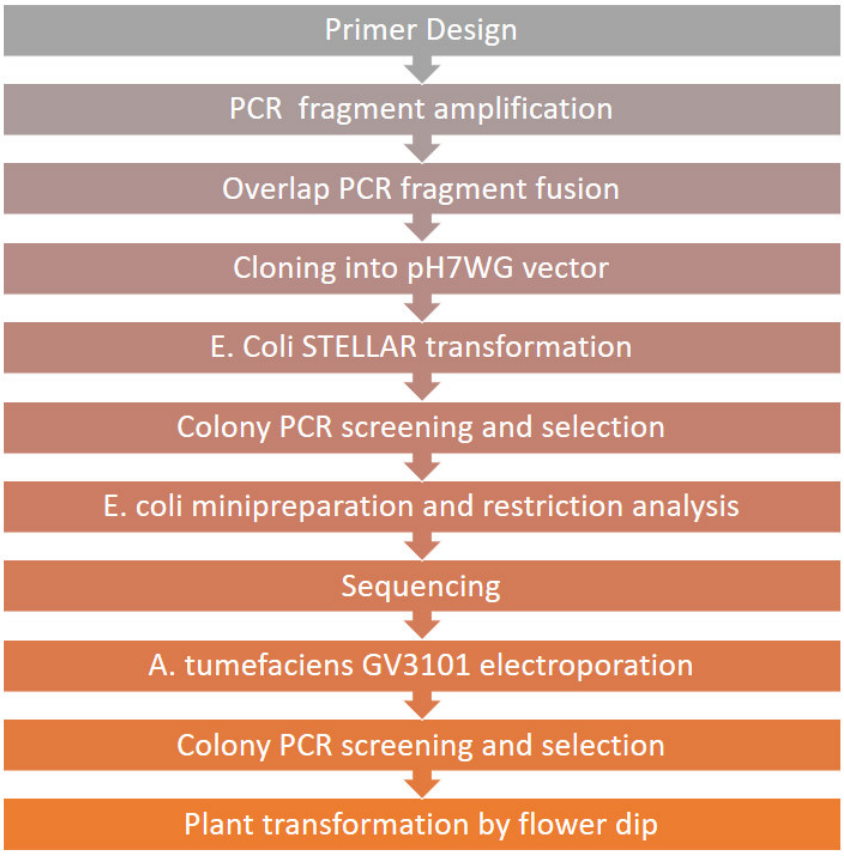

Figure 11 - Flowchart representation of the steps performed for obtaining, cloning and screening the desired constructs

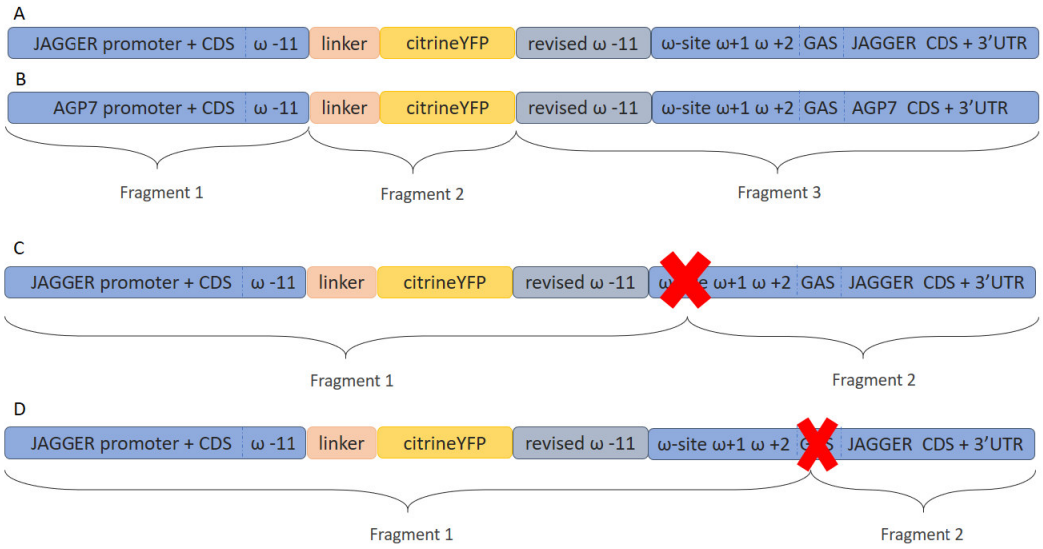

Figure 12 - Schematic representation of the cYFP fusions planned in this work and detailed in this section: A, B - nascent AGPs; C - JAGGER with deleted omega-site; D - JAGGER with deleted hydrophobic domain.

The genomic, full length, JAGGER and AGP7 sequences were obtained from healthy *Arabidopsis* DNA by PCR amplification using the primers listed in Table I. The constructs were designed (supplemental images 2 and 6) according to previous GPI-anchored proteins (GAP) cYFP constructs studied by Liu *et al.* (2016), who successfully reported subcellular localization in *Arabidopsis* ovules.

This design is represented in figure 12 and consists of three fragments: AGP N-terminal sequence up to 11 amino acids upstream of the ω-site; the citrine Yellow Fluorescent Protein (cYFP); a revised ω-11 sequence followed by the ω-site and the C-terminal end of the protein. The revised ω-11 region was included in case any regulatory elements needed by the ω-site are present in this region, but coded for the same amino acids using alternate codons, observing *Arabidopsis* codon usage bias, to prevent over enhancement, should that be the case.

2.1.1. Obtaining JAGGER GPI-signal mutants

Three GPI-signal mutants were prepared: JAGGERΔω-site, JAGGERΔ2ω and JAGGERΔGAS (supplemental images 3,4 and 5).

Once the JAGGER-cYFP plasmid was ready, it was used as a template to amplify the fragments for the GPI signal mutants. The deletion was incorporated in the primer sequence used, as detailed in table II.

Table I: Primer sequences used to amplify JAGGER-cYFP and AGP7-cYFP fragments		
Name	**Sequence**	**Description**
JAGGER::cYFP - 1	ggcggccgcactagtctggtaatatttgataaaagcgg	Anneals in JAGGER promoter; Includes a 15 bp overlap with pH7WG vector (highlighted in red); Used in fragment amplification and overlap PCR. (Fw)
JAGGER::cYFP - 2	GGCCTGGGGCAGGGGATGCATCTGA	Anneals in w-11 region; Includes a 15 bp overlap with primer JAGGER::cYFP-3 (red); Used for fragment amplification. (Rv)
JAGGER::cYFP - 3	CCCCTGCCCCAGGCCGGCCTGGAGGTGGA	Anneals in linker; Includes a 15 bp overlap with primer JAGGER::cYFP-2 (red); Used for fragment amplification (fw)
JAGGER::cYFP - 4	TGGAGAAGCGTCAGATGGACCAGGTGCCTTGTACAGCTCGTCCATG	Anneals in revised w-11; Includes a 19 bp overlap with cYFP (red); Used in fragment amplification and overlap PCR. (Rv)
JAGGER::cYFP - 5	TCTGACGCTTCTCCAGCACCTAGCGCCGCATTCTCC	Anneals in JAGGER coding sequence; Includes a 15 bp overlap with primer JAGGER::cYFP-4; Used for fragment amplification (fw)
JAGGER::cYFP - 6	ctgggtcggcgcgccatgtttaagtgaaagagtaatgaaattgat	Anneals in JAGGER 3'UTR; Includes a 15 bp overlap with pH7WG vector; Used for fragment amplification and overlap PCR (Rv)
AGP7::cYFP - 1	ggcggccgcactagttagctctcttctcaggtttc	Anneals in AGP7 promoter; Includes a 15 bp overlap with pH7WG vector (red); Used in fragment amplification and overlap PCR. (Fw)
AGP7::cYFP - 2	TCCAGGCCGGCCAGGTGGAGGAGCGGAA	Anneals in w-11 region; Includes a 15 bp overlap with primer AGP7::cYFP-3 (red); Used for fragment amplification. (Rv)
AGP7::cYFP - 3	TTCCGCTCCTCCACCTGGCCGGCCTGGA	Anneals in linker; Includes a 15 bp overlap with primer AGP7::cYFP-2 (red); Used for fragment amplification (Fw)
AGP7::cYFP - 4	GATGCGTCAGATGGAGTAGGCTTGTACAGCTCGTCCATG	Anneals in revised w-11; Includes a 15 bp overlap with cYFP (red); Used in fragment amplification and overlap PCR. (Rv)
AGP7::cYFP - 5	TCCATCTGACGCATCAGCACCACCTCCAAACGCCGCTTTAACCA	Anneals in AGP7 coding sequence; Includes a 15 bp overlap with primer AGP7::cYFP-4; Used for fragment amplification (Fw)
AGP7::cYFP - 6	ctgggtcggcgcgccgagaatacctgattttataa	Anneals in AGP7 3'UTR; Includes a 15 bp overlap with pH7WG vector; Used for fragment amplification and overlap PCR (Rv)

Table II: Primer sequences used to amplify the modified JAGGER fusions (JAGGERΔωsite-cYFP, JAGGERΔ2ω-cYFP and JAGGERΔGAS-cYFP) fragments		
Name	**Sequence**	**Description**
Δω-site - 1	AGAATGCGGCAGGTGCTGGAGAAGCGTC	Pairs with JAGGER::cYFP – 1 (Fw) Anneals in w-11 region and incorporates deletion of the highest predicted ω-site (Rv)
Δω-site - 2	AGCACCTGCCGCATTCTCCAACAAGG	Anneals in w-11 region and incorporates deletion of the highest predicted ω-site (Fw) Pairs with JAGGER::cYFP – 6 (Rv)
Δ2ω - 1	GGAGAATGCGGCAGGTGCTGGAGCGTC	Pairs with JAGGER::cYFP – 1 (Fw) Anneals in w-11 region and incorporates deletion of the two putative ω-site predictions (Rv)
Δ2ω - 2	GACGCTCCAGCACCTGCCGCATTCTCCA	Anneals in w-11 region and incorporates deletion of the two putative ω-site predictions (Fw) Pairs with JAGGER::cYFP – 6 (Rv)
ΔGAS - 1	taaaaaaagttcTCAAGCCTTGTTGGAGAATGCGGCGCT	Pairs with JAGGER::cYFP – 1 (Fw) Anneals in CDS+ 3'UTR and incorporates deletion of the hydrophobic GAS domain (Rv)
ΔGAS - 2	AGCACCTAGCGCCGCATTCTCCAACAAGGCT	Anneals in CDS+ 3'UTR and incorporates deletion of the hydrophobic GAS domain (Fw) Pairs with JAGGER::cYFP – 6 (Rv)

2.2. *agp7* reproductive phenotype analysis

Seed set Scoring

Siliques from mature *Arabidopsis* wild-type and *agp7* -/- plants (stage 17-18 according to Smyth *et al.*, 1990) were selected according to pattern placement: between the 5th and the 20th silique from a main stem, we chose 5 siliques from distinct stems of the same individual, and repeated this for 5 distinct individuals. Silique was excised, placed in a microscope slide, a scalpel was used to open the valves and the seeds inside were manually counted and divided into two categories: viable and aborted.

Controlled Pollinations

Arabidopsis agp7 -/- flowers, and wild-type flowers were chosen according to their developmental stage (before bud opening - stage 12 according to Smyth *et al.*, 1990) in order to guarantee no self-pollination had already occurred. The flowers were emasculated by severing their anthers, after removing siliques, buds and open flowers from the plant under a stereomicroscope. Each pistil, stripped of surrounding sepals and petals by use of hypodermic needles (0.4 x 20 mm; Braun), was enclosed in cling film for protection and to prevent dehydration. The emasculated flowers were afterwards hand pollinated, by removing a stamen from the pollen donor plant and rubbing the

anther against the stigma: *agp7* pistils were pollinated with wild-type pollen and wild-type pistils with *agp7-/-* pollen. When the stigma was well coated with donor pollen, it was again enclosed in cling film to prevent undesired pollination, and to provide protection and humidity to the now naked pistil.

Aniline Blue staining

The resulting pistils from reciprocal *agp7-/-* x wild-type crosses were collected and fixed in absolute ethanol and glacial acetic acid in a 9:1 ratio, and left overnight (ON) at 4ºC. They were then washed 3 times in dH2O for 5 minutes and left ON in an NaOH solution (7,5-8 M) to bleach the tissues. Another 3 washes in dH2O followed, for 20 minutes each. Afterwards the pistils were stained in a Decolorized Aniline Blue Solution (DABS) (0,1% Acid Blue, Sigma in 100mL K3PO4 0,1M), and kept ON at 4ºC.

Preparation of plant material for observation and cell imaging

In order to observe the pollen tube *en route* to the embryo sac, plant material was collected approximately 16h after pollination. The pistils were placed in microscope slides, and using a stereomicroscope (model GZ4; Leica) and hypodermic needles (0.4 x 20 mm; Braun), the protective valves were removed to expose the septum and the ovules. The desired tissues were then mounted in water for observation. Cell imaging was obtained in an Inverted Microscope (Eclipse Ti-S; Nikon) with UV fluorescence.

2.3. Obtaining a double homozygous *jagger agp7* mutant

The mutant lines *jagger* -/- and *agp7* -/- were crossed employing the controlled pollination technique already described in the previous subchapter 2.2. The resulting seeds from this cross were collected, sown, and allowed to develop into adult plants. The plants grown from these seeds are currently under screening for a double homozygous individual.

2.4. Molecular biology protocols

Bacterial Strains and growing conditions

Different bacterial strains were used in the course of this work, either for plasmid maintenance or to obtain expression in plant cells: *Escherichia coli* STELLAR strain, and *Agrobacterium tumefaciens* strain GV3101::pMP90. Bacterial growth took place in Luria-Bertani medium (LB) [10 g tryptone, 5 g yeast extract, 10 g NaCl for every 1 L of medium;

for solid medium 1.5% (w/v) microagar was added (LB Agar)] at 37 ºC for *E. coli* and 28 ºC for *A. tumefaciens*. Liquid cultures were grown under orbital agitation. Selection for the plasmid of interest was made by supplementing the LB medium with 50 µg/mL antibiotic (spectinomycin for pH7WG). When working with *A. tumefaciens*, 20 µg/mL gentamycin was also added to maintain selective pressure on the helper plasmid resistance.

InFusion Cloning – Insertion into plasmid vector and transformation of competent E. coli

For this work the shuttle vector Ph7WG was selected, which allows to propagate the plasmids in different cell types: it can be amplified and maintained in *E. coli*, and expressed in *A. tumefaciens* and *A. thaliana*. In Fusion Cloning is a method for recombinational cloning that requires no ligation – simply 15bp overhangs between the fragments to be fused together. The In-Fusion Cloning (Clontech) kit was used, according to manufacturer's instructions. An overview of the protocol is demonstrated in figure 13.
Competent *E.coli* Stellar cells were also made available on the Clontech kit.

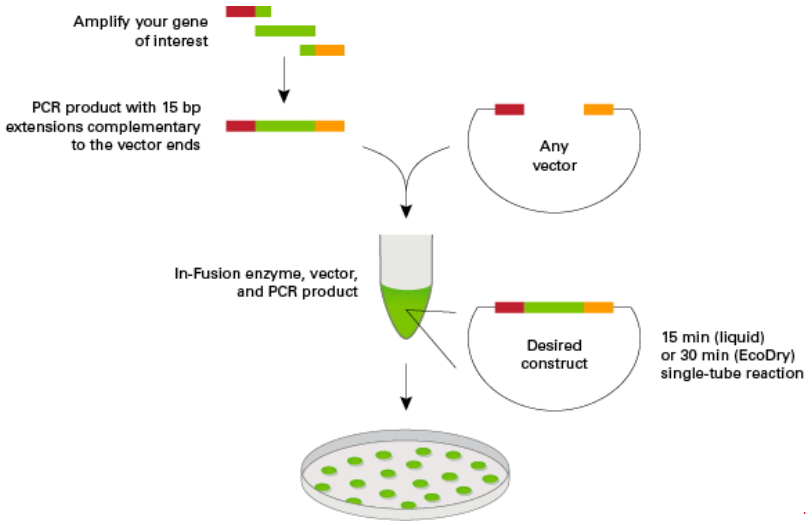

Figure 13 - Overview of the In Fusion Cloning ligation-independent method

Agrobacterium tumefaciens Transformation by Electroporation

An aliquot of electrocompetent *A. tumefaciens* GV3101::pMP90 was thawed on ice. A new electroporation cuvette was placed to cool on ice for 30 minutes. To these cells,

5 µL of pure plasmid DNA were added, and the entire volume transferred into the cuvette. Using a pre-programmed "*A. tumefaciens*" mode in the Biorad Micropulser, an electrical shock was delivered to the cells and 1 mL of LB medium was quickly added. The cells were allowed to recover for a 4h period without agitation at 28 ºC in a 1,5 mL tube. A centrifugation of 4 minutes at 1.100 x g followed, the supernatant was discarded and the pellet ressuspended in 100 µL of LB medium. This volume was plated in LB-agar supplemented with proper antibiotics, and the plates were incubated for 48h at 28 ºC with agitation.

Plasmid DNA extraction

Plasmid DNA was extracted using the "QIAprep® Miniprep Kit" (Quiagen) according to the manufacturer's instructions.

Plasmid DNA Digestion

Plasmid DNA digestion intended for restriction analysis was performed according to the restriction enzymes manufacturer's instructions (New England BioLabs, NEB).

Genotyping

A PCR-based approach was performed in both *agp7* and *jagger* mutant lines, in order to guarantee we were working with homozygous mutant plants, by confirming the presence of the T-DNA insertion in each individual plant. Specific genotyping primers were used: LP-GK-134A10, RP-GK-134A10 and 08409 for *jagger*; for the *agp7* mutant line the LP-SALK-039285, RP-SALK-039285 and LBb1.3 were used. Primer sequences are listed in Table III.

Two distinct reactions were prepared for each mutant:

- One, pairing Left Primer (LP) and Right Primer (RP), which anneal in the genomic sequence and will result in an amplified fragment only if T-DNA is absent – thus identifying the plant as wild-type for the locus;

- Another, pairing a T-DNA Border Primer (BP - 08409 or LBb1.3), which anneals in the Left Border of the T-DNA insertion sequence, and RP primer which will only yield an amplified fragment in the presence of the T-DNA insertion.

Analysing these PCR products by agarose gel electrophoresis it is possible to distinguish a homozygous (HM) mutant plant, from a heterozygous (HZ) plant, and from a wild type (WT) plant, according to the band pattern obtained. The reaction and conditions used for genotyping are stated in tables IX and X, respectively.

Table III: Primer sequences used for genotyping *jagger* and *agp7* plants

Name	Sequence	Description
213.LP-GK-134A10 (*jagger*)	TGTCTCCCCACATTTGCCAT	Anneals in JAGGERs promoter region (Fw)
129.pAC161GK (Gabi-Kat)	ATATTGACCATCATACTCATTGC	Anneals in GK lines T-DNA insertion (Fw)
214.RP-GK-134A10 (*jagger*)	ACAACCATATGAAGCCCTTCC	Anneals in JAGGERs 3'UTR region (Rv)
215.LP-Salk-039285 (*agp7*)	ACTGGACAGATCTCAGATCCG	Anneals in AGP7s promoter region (Fw)
LBb1.3 (SALK)	ATTTTGCCGATTTCGGAAC	Anneals in SALK lines T-DNA insertion (Fw)
216.RP-Salk-039285 (*agp7*)	TTCACTTAAACGTTTCTGCTATTTG	Anneals in AGP7s 3'UTR region (Rv)

DNA Gel Electrophoresis

Sample DNA analysis was made in 1,0% (w/v) agarose gel in 1X TAE buffer [40 mM Trizma-base, 10% (w/v) glacial acetic acid and 10 mM EDTA], with 0,5 mg/mL Ethidium Bromide added before polymerization. 1X loading dye [15% (w/v) Ficol 400 and 0,25% (w/v) Bromophenol Blue] was added to each sample prior to loading. Using the "GeneRuler DNA Ladder Mix" (Thermo Fisher Scientific) as molecular weight marker and 1X TAE running buffer, the electrophoretic separation was conducted at 150 V and non-limiting amperage for 40 minutes. Ethidium bromide fluorescence allowed DNA visualization in a UV transilluminator (302-365 nm).

DNA purification from agarose gel

To prevent DNA damage, desired bands were swiftly excised at lowest UV retro illumination available. A "E.Z.N.A.® MicroElute Gel Extraction Kit" (Omega) was used to extract the DNA from the agarose gel, as instructed by the manufacturer.

DNA Quantification

DNA was quantified by use of a NanoDrop Spectrophotometer ND-1000, following the manufacturer's instructions.

Polymerase Chain Reaction

To amplify the desired fragments from either genomic DNA (JAGGER and AGP7) or plasmid DNA (cYFP), the high fidelity "PrimeSTAR® GXL DNA Polymerase" (Clontech) was used to avoid amplification mistakes. Table IV indicates how these reactions were

performed. The "DNA engine Dyad Peltier Thermal Cycler" (Biorad) was programmed according to the conditions stated in table V.

Table IV - PCR reaction for high fidelity cloning (fragment amplification)

Reagents	Concentration used
Template DNA	500ng – 1 µg
10x GXL buffer	1x
dNTPs	0.2 mM
Primer forward	0.3 mM
Primer reverse	0.3 mM
GXL enzyme	1U
ddH_20	Up to 50 µL

Table V –High fidelity cloning (fragment amplification) conditions

Step		Temperature/Duration
Initial denaturation		94°C / 3 min
30 cycles	Denaturation	94°C / 30 sec
	Annealing	55°C / 30 sec
	Extension	68°C / 1 min per KB
Final extension		68°C / 10 min

Site-Directed Mutagenesis

Upon obtaining JAGGER-cYFP construct and verifying it by sequencing, the plasmid was used as template to obtain the mutated JAGGER versions. The reaction and its conditions occurred according to tables VI and VII, respectively. In each of the three modified versions, the mutation was induced by the modified primers sequence (Table II), which caused a deletion in the final product.

Table VI - PCR reaction for site-directed mutagenesis

Reagents	Concentration used
Template DNA	20 – 50 ng
10x GXL buffer	1x
dNTPs	0.2 mM
Primer forward	0.3 mM
Primer reverse	0.3 mM
GXL enzyme	1U
ddH_20	Up to 50 µL

Table VII –Site-directed mutagenesis conditions

Step		Temperature/Duration
Initial denaturation		94°C / 3 min
20 cycles	Denaturation	94°C / 30 sec
	Annealing	58°C / 30 sec
	Extension	68°C / 1 min per KB
Final extension		68°C / 10 min

«Colony PCR» Screening

The screening for positive colonies was performed by «colony PCR», a technique that allows confirmation of both the inserts presence. The primers used were the same for both proteins, as they amplify a cYFP segment, which is present in all constructs (Table VIII). PCR reaction was assembled on ice according to table IX. A sterile toothpick was used to touch the desired colonies and then washed in the prepared PCR tubes. PCR

conditions were as described in Table X, using the enzyme "OneTaq® DNA Polymerase" (NEB).

Table VIII: Primer sequences used for colony PCR screening

Name	Sequence	Description
1403 – citrine	AAGCTGACCCTGAAGTTCATCTG	Anneals in cYFP region (Fw)
1394 – citrine	GCCGATGGGGGTGTTCTG	Anneals in cYFP region (Rv)

Table IX - PCR reaction for genotyping and colony PCR

Reagents	Concentration used
Template DNA	500 ng - 1µg
10x OneTaq buffer	1x
dNTPs	0.4 mM
Primer forward	0.2 mM
Primer reverse	0.2 mM
OneTaq enzyme	1U
ddH_20	Up to 25 µL

Table X – Genotyping and colony PCR conditions

Step		Temperature/Duration
Initial denaturation		94°C / 3 min
35 cycles	Denaturation	94°C / 30 sec
	Annealing	59°C / 30 sec
	Extension	72°C / 1 min per KB
Final extension		72°C / 10 min

Overlap PCR

This technique creates long DNA fragments from shorter ones. As illustrated in figure 14, overlap PCR takes place along two distinct phases: the first phase consists in obtaining the desired DNA fragments, by independent classical PCR reactions, with the primers listed in table I.

The resulting fragments will contain overlapping regions with each desired/planned flanking sequence. The second phase consists in bringing all the fragments together: fragment one and two were brought together by a PCR reaction as described in table IV, to which only primer 1 and primer 4 are added: this way only the fragments that have aligned according to the overlapping regions are amplified.

Fragment 1+2 and 3 were brought together by a PCR reaction as described previously, this time adding only primer 1 and primer 6. This last reaction will yield a single insert composed of fragment 1+2+3, flanked by overhangs, which will overlap with the vector, on each side.

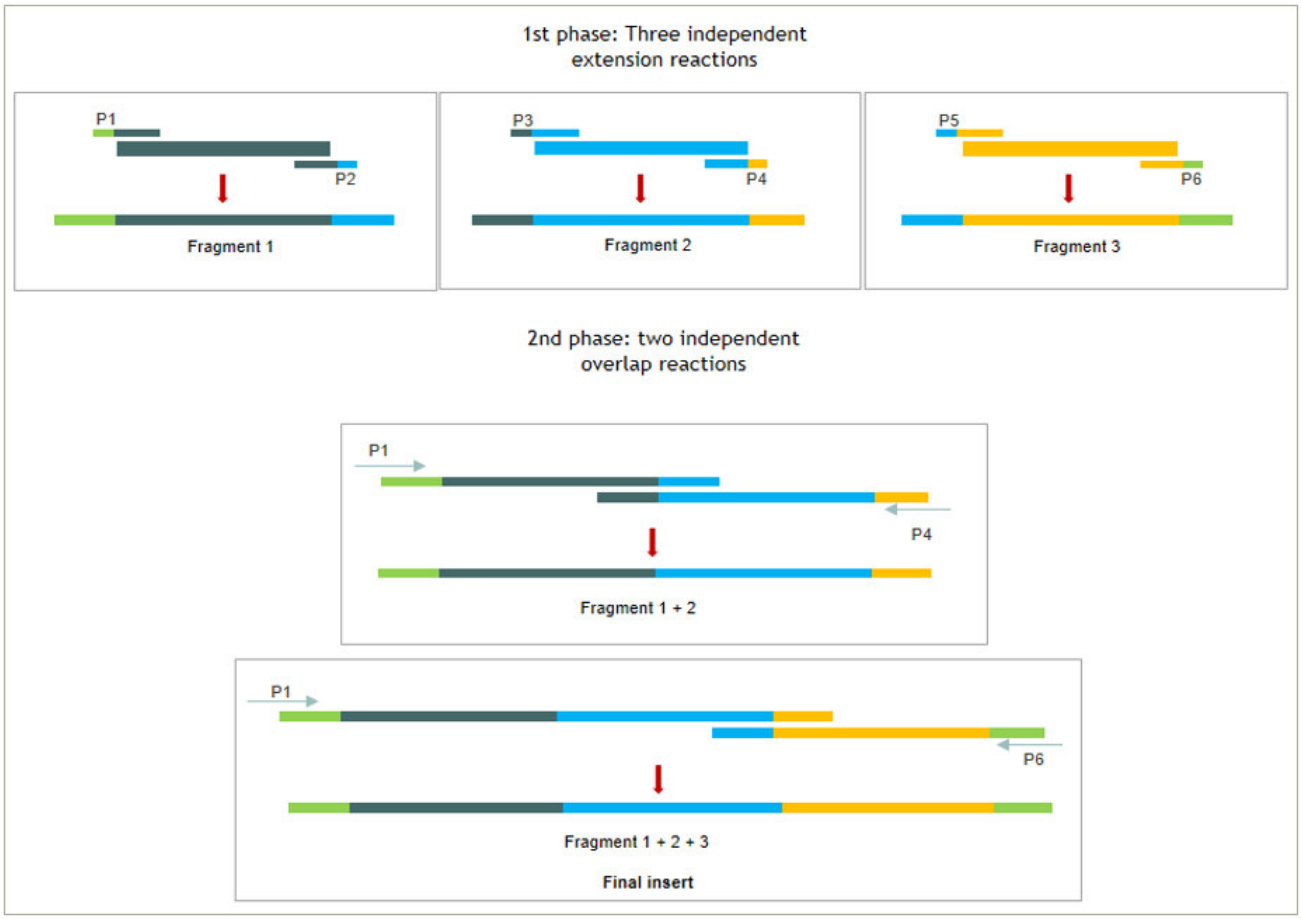

Figure 14 - Schematic representation of an Overlap PCR.

Arabidopsis thaliana genomic DNA extraction

Tissue was collected from the rosette of healthy, young plants (1 to 3 leaves), inserted in a 1.5mL Eppendorf tube containing 4-5 metallic beads. The tube was placed in a grinder for 90sec and then centrifuged at 12xg for 1min. In the chemical hood, 400uL extraction buffer (100uL glycogen 1mg/mL; 20mL 1M Tris-HCl pH 9.0; 20mL 2 M LiCl; 5mL 0.5 M EDTA pH 8.0; 10mL 10% SDS; 500uL B-Mercaptoethanol; 44.4mL H_2O) was added and the tube inverted. A centrifugation at 12xg for 2 min followed. 400uL phenol/chloroform (pH8.0) was added, and the tube vortexed to assure thorough mixing. Sample was then allowed to sit for 5 minutes, followed by a centrifugation at 12xg for 10 min. Supernatant was carefully moved to a new tube, and 400uL isopropanol added, inverting the tube 5-6 times, and placed at -20ºC for 15 min. Sample was then centrifuged at 12xg for 15 min and the supernatant discarded. The resulting pellet was washed with 750uL 70% EtOH, centrifuged at 12xg for 5 min and supernatant removed. This was

followed by a 10 secs spin. Removal of residual EtOH was done by pipetting and allowing the tube to sit with open lid for 5 minutes to evaporate. DNA was then ressuspended in 50uL ddH_2O and stored at -20ºC.

2.5. Biological material - maintenance and transformation

Nicotiana benthamiana system

In order to test the efficiency of the cloning and the functionality of the fusions *in planta*, a transient expression assay was performed in *Nicotiana benthamiana*, based on an Agrobacterium infiltration method in leaves, already established in Palanivelus Lab.

Arabidopsis thaliana system

The fusion proteins were used to produce stable transgenic *Arabidopsis* lines for each construct, in order to obtain a constitutive expression system, using an optimized floral dip method adapted from Clough and Bent (1998).

Plant Materials and Growth Conditions

All seeds used belong to either the *Arabidopsis thaliana* Columbia (Col-0) or Nossen (No-0) ecotypes. Mutant lines *jagger* -/- and *gpi8-2*/+ were reported previously [Pereira *et al.*, 2016; Liu *et al.*, 2016, respectively]. Mutant line *agp7* -/- (SALK_039285) was obtained from the European *Arabidopsis* Stock Centre (NASC).

Seeds were surface sterilized by gas sterilization and plated on Murashige and Skoog plates, containing the corresponding antibiotics, when applicable. Plated seeds were stratified at 4°C for 3 days and then placed on the growth chamber maintained at 20°C and continuous light. When seedlings were 7 to 10 days old, they were transferred to individual soil pots and were grown inside a chamber (Fig.15) with a programmed day night cycle of 16 hours light at 21°C and 8 hours darkness at 18°C.

Figure 15 - *Arabidopsis* plants in growth chamber.

Arabidopsis thaliana transformation by floral dip

Healthy *Arabidopsis* plants, with mature, properly developed siliques, and the highest number of flowering buds containing flowers around stage 12 [Smyth *et al.,* 1990], were selected for transformation. *A. tumefaciens* containing the desired plasmids were incubated in 5mL LB medium supplemented with the adequate antibiotics, and allowed to grow in a 28ºC orbital agitation incubator. 24h after, 4 mL of this culture was added to 400mL liquid LB (adequately supplemented) and again allowed to grow in the same conditions for 16h. This liquid culture was then transferred to a centrifuge bottle (figure 16), centrifuged at 6.000g for 20min at 4ºC and the supernatant discarded. The resulting pellet was suspended in 200mL transformation medium (2.15g MS; 50g Sucrose, 0.5g MES; pH adjusted to 5.8 with KOH; 10 µg 6-BA, 200µL Silwet-77).

All flowers/siliques older than stage 12 were excised and discarded, to increase the transformation efficiency. Each construct was transformed into 15 plants. Dipping was repeated every 4 days, using freshly prepared media and Agrobacterium cultures, to a total of 5 times per construct.

After transformation, plants were allowed to fully mature, and seeds from siliques were collected.

Figure 16 - Floral dip setup. 1: Plants to be transformed; 2: centrifuge bottle containing the desired Agrobacterium colony, suspended in transformation medium; 3: Petri dish where the dipping of individual flowers is performed; 4: liquid waste beaker.

Gas Seed Sterilization

The desired seed quantity was measured into a 1.5mL Eppendorf tube with a circular puncture of approx. 1mm diameter in its lid. The Eppendorf was placed inside a glass sterilization chamber, together with a beaker containing 95mL bleach and 5mL HCl, and kept for 3 hours. This procedure took place inside the chemical hood. This method was used for all seeds prior to sowing, except seeds resulting from floral dip.

Liquid Seed Sterilization and Plating

Using a 1.5mL Eppendorf tube, approximately 0.1mL of seeds were measured and transferred to a 15mL falcon tube. 10mL of bleach solution was added to the falcon tube, inverted 2-3 times to soak the seeds, and then allowed to sit for 7-8 minutes. In the laminar flow hood, the bleach solution was discarded and 10mL sterile water was added, the tube inverted to wash the seeds, and after allowing the seeds to settle the water was discarded. This washing step was repeated 3 more times. Afterwards, 10mL of MS media was added to the tube, gently swirled to assure all seeds are suspended in the media, and then swiftly poured onto large MS plates, supplemented with hygromycin. The plates were not fully covered with the lid until agar was dry, to avoid condensation. Once ready, plates were kept in 4ºC in the dark for 3 days. This method was used only when screening transformant seeds.

T1 Transformant Seeds Screening

After the plated seeds to be screened have been in the dark at 4ºC for 3 days, they were placed in the growth chamber (long days) for 5-6 hours and afterwards placed in the dark at room temperature for another 3 days. Finally, the plates where placed in the growth chamber and monitored. The first hygromycin resistant seedlings were noticeable and ready to be transplanted approximately 4 weeks after.

Transient expression in Nicotiana benthamiana

With the purpose of qualitative validating the constructs functionality, we prepared in parallel a transient expression assay. *Nicotiana benthamiana* was chosen as heterologous system due to a simple and already well-established protocol for *Agrobacterium* mediated infiltration, which is easily reproduced, requires no specific equipment and allows for rapidly observable transient expression in large mesophyll leaves. In this work, three weeks old plant leaves were chosen for infiltration.

Preparation of the inoculum

Two *Agrobacterium* liquid cultures, one carrying the desired plasmid to transform and another with the helper plasmid, were initiated: 2mL LB media, containing the appropriate antibiotics, was inoculated and incubated in an oscillating incubator at 28º for 24h (if from glycerol stock) or 16h (if from streak plate). On the following day, 1mL of culture was harvested and centrifuged at 5xg for 5 mins. The supernatant was discarded, the pellet ressuspended in 1mL of $MgCl_2$ (10mM solution), and centrifuged on the same conditions stated. Again the supernatant was discarded and the pellet ressuspended, this time in *Agrobacterium* induction Media (0.1M MES buffer at 5.6pH; 1M $MgCl_2$; 25mM acetosyringone dissolved in DMSO) and left to incubate at room temperature for 2h.

A 1/5 dilution of the sample was prepared in order to measure optical density in a spectrophotometer. This allowed us to prepare the final 1 mL samples to infiltrate, according to the formula: [(Desired OD600/OD600 of dilution) x 1000] x 1/5.

Syringe-mediated leaf infiltration

A 20uL pipette tip was gently rubbed on the abaxial side of a chosen, fully developed leaf, to induce a tear in the leaf's surface (avoiding puncturing the leaf from one side to the other). The aperture should be small enough for the syringe to cover completely when placed upon.

The infiltration was performed with a syringe without needle, applying pressure to the opening previously created, as demonstrated in fig. 17. The plant was then returned to the growth cabinet before observation.

Figure 17 - *Nicotiana benthamiana* leaf infiltration.

3. Results

3.1. Phenotypic analysis of *agp7* -/- mutant plants

jagger -/- is a sporophytic mutant whose known reproductive phenotype manifests in the increase of polytubey occurrence. It has been demonstrated that this happens due to JAGGER being essential for persistent synergid degeneration, this cell being the source of the attractant molecule that keeps on "calling" pollen tubes, even after successful fertilization [Pereira *et al*., 2016]. However, this mutation is not fully penetrant, which points out the possibility of another player involved in the process. AGP7 is a protein with a high degree of similarity with JAGGER, which led us to consider it a good candidate for this role.

Seed set scoring

To analyse a possible reproductive mutant, we looked into the direct output of reproductive success: the amount of seed yielded. Here we analysed the seed set of agp7 -/- mutant plants and compared it to the seed set of wild-type plants sewn, grown, and developed at the same time and under the same conditions. We report here that no significant difference was found.

Aniline Blue Staining

Callose, a β 1, 3-glucan, is one of the main components of the pollen tube cell wall. Addicionally, pollen tubes periodically produce callose plugs as they advance, as a measure to keep all cytoplasm near the growing tip. Aniline blue is a callose-specific fluorochrome, and as such labels the pollen tube, from the moment it protrudes from the grain until it penetrates the ovule. Aniline blue preparations show bright blue fluorescence under fluorescence microscope with UV illumination. [Kho & Baër, 1968; Franklin-Tong, 1999].

When analysing a mutant phenotype, this technique is useful to determine pistil's receptivity and viability as well as the pollen correct development. In the particular case of this study, it is invaluable to observe the pollen tube growth towards the ovule, and if

polytubey occurrence is within normal wild-type, values, or if there is a noticeable increase. In figure 18 we can observe representative images of pollen tubes, whose journey through the pistil tissues was successful, entering the embryo sac of wild-type (A) and *agp7* (C) ovules, respectively, and of a thoroughly pollinated *agp7* stigma (B). The resulting pistils of 6, independent, reciprocal crosses were observed.

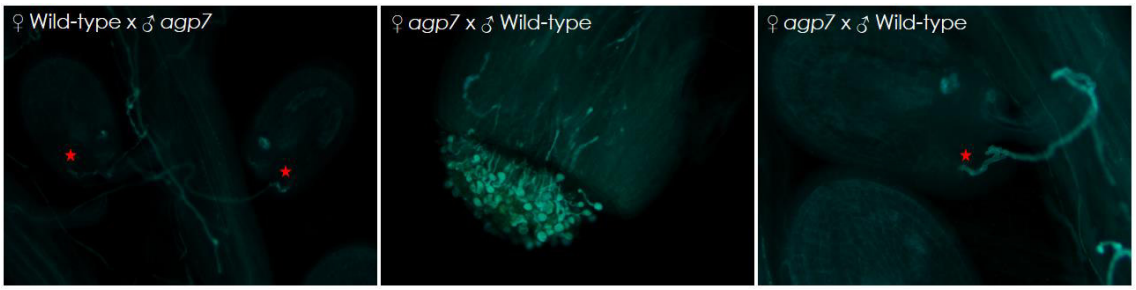

Figure 18 - Visualization of aniline blue stained pollen tubes. *A: Wild-type pollen tubes (marked with red stars) entering agp7 -/- ovules: a single pollen tube is matched to each ovule. ; B: agp7 -/- stigma covered with pollen grains and respective pollen tubes growing along the style. C – a single agp7 -/- pollen tube (red star) entering a wild-type ovule.*

3.2. Achieving double homozygous mutant *jagger/agp7*

Although a double homozygous mutant *jagger/agp7* was already available in Professor Coimbra's' Lab, it was observed that beyond first generation the double mutant displayed inconsistencies. We chose to prepare a double mutant anew to carefully study each generation and determine if this variation is due to any anomaly of this particular mutant.

As stated earlier, *jagger* single mutant phenotype was not fully penetrant, so thoroughly studying the double *jagger agp7* double mutant and observing if the polytubey phenotype is present to a higher degree is a pivotal step in unravelling these proteins putative intertwined roles. In order to achieve this, *jagger* and *agp7* mutant plants were chosen, their genotype confirmed by PCR (Fig. 19), and hand pollination was performed in order to cross them.

The resulting seeds were collected, planted and allowed to grow. Seed was collected from the resulting heterozygous plants, planted, grown and are being screened at the time this chapter is written.

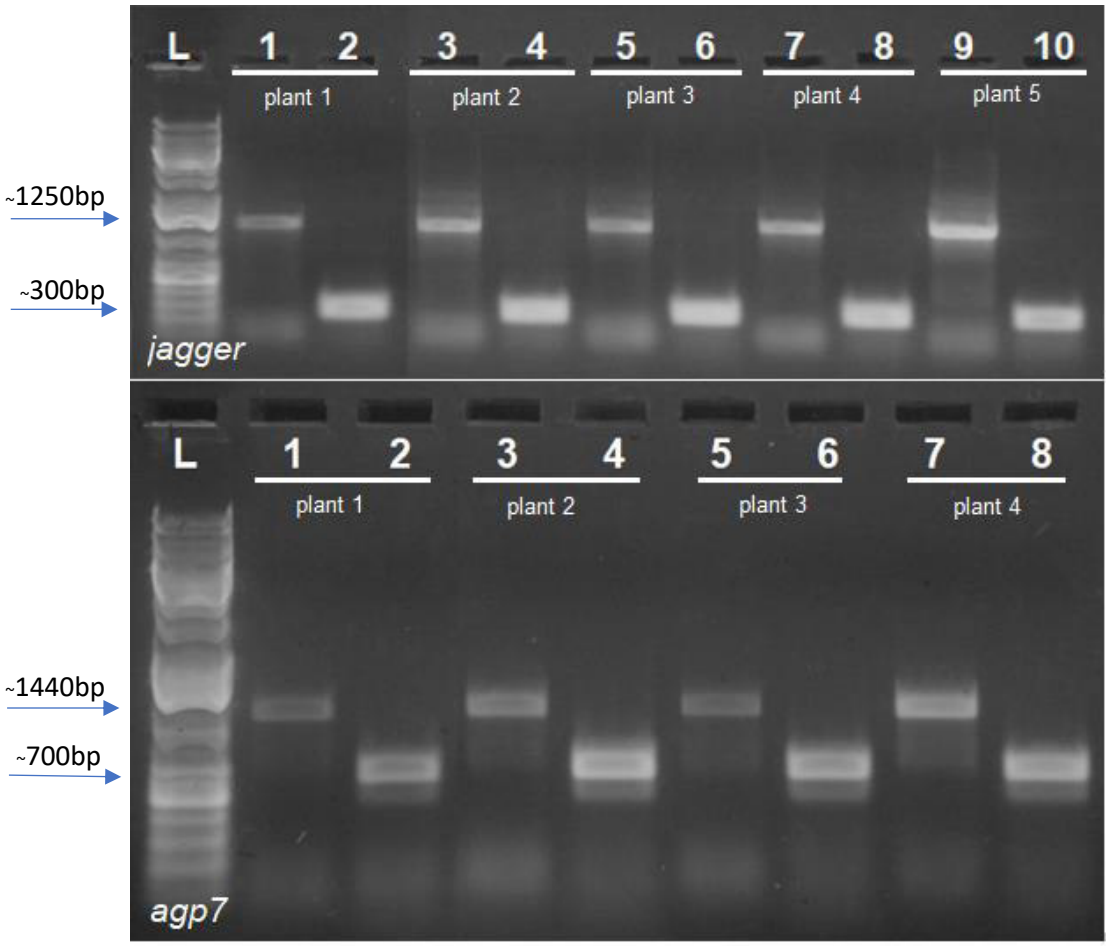

Figure 19 - Electrophoretic screening of F1 double *jagger agp7* mutants by genotyping.
L: Molecular weight marker; even numbers: wild-type band; odd numbers: mutant band.

3.3. Subcelullar Localization of JAGGER and AGP7

To study JAGGER and AGP7 subcellular localization, and to test mutant phenotype complementation, we first obtained constructs containing the native promoter and coding sequence for each of the proteins, fused to a citrine YFP reporter. To study the influence (in both function and localization) of specific sites within the GPI signal contained in the nascent JAGGER protein, mutated versions of JAGGER-cYFP, described in detail in subchapter 3.3.2, were also prepared. To express the constructs in *A. thaliana* they were inserted in the binary expression shuttle vector, pH7WG.

3.3.1. Obtaining JAGGER/AGP7-cYFP constructs

Fragment amplification

The first step is amplifying the three independent fragments planned for each construct, as we have seen in figure 14. We do so by conducting individual PCR reactions, using the specific primers stated in table I. Each PCR product was analysed by agarose gel electrophoresis (Figs. 20 and 21). The bands whose molecular weight corresponded to the desired fragment were excised from the gel and purified.

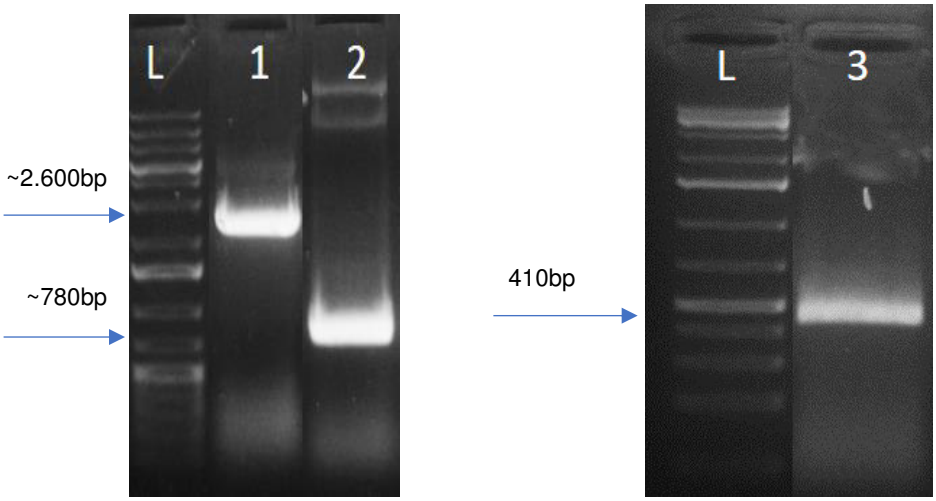

Figure 20 – Electrophoretic analysis of the amplified JAGGER-cYFP fragments. *L – DNA molecular weight marker; 1- Fragment 1; 2- Fragment 2; 3- Fragment 3.*

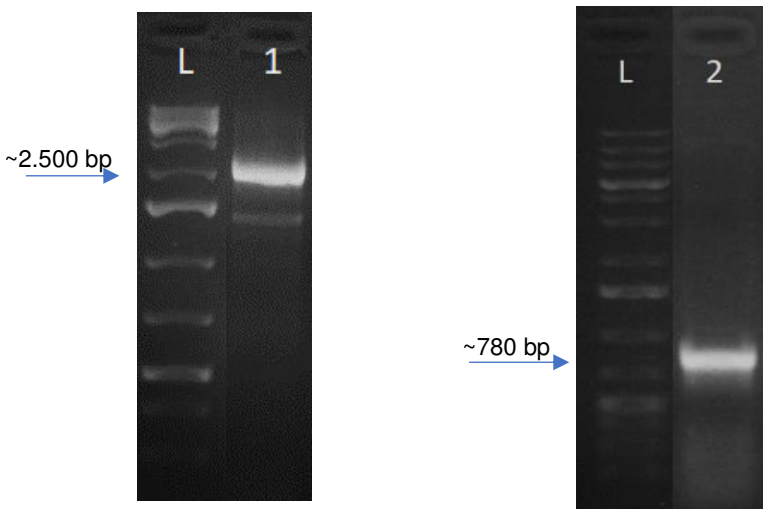

Figure 21 - Electrophoretic analysis of the amplified AGP7-cYFP fragments. *L- DNA molecular weight marker; 1 – fragment 1; 2 –fragment 2.*

Due to unfortunate high complementarity in AGP7-cYFP 3rd fragment and its adjacent vector segment, we were unable to amplify this fragment. After several attempts at optimization, primer/template ratios, touchdown PCR, DMSO, we opted to have this fragment synthesized by IDT - Integrated DNA Technology.

Creating a single insert

The independent fragments where brought together and made continuous by overlap PCR, taking advantage of the complementary overhangs each fragment extremities possess. The technique is detailed and illustrated in chapter 2, figure 14.

In both *JAGGER* and *AGP7*, we first created the fused fragment 2+3, these being the smallest in length, analysing the PCR by agarose gel electrophoresis and purifying the fragment with the expected size from the gel. A second overlap reaction followed, to bring fragment 1 together with this fused fragment 2+3.

Again, the PCR product was analysed by electrophoresis, and the desired fragment purified from the gel (fig. 22). In the end, we obtained a single, continuous 1+2+3 fragment for each construct, ready to be inserted into the planned vector.

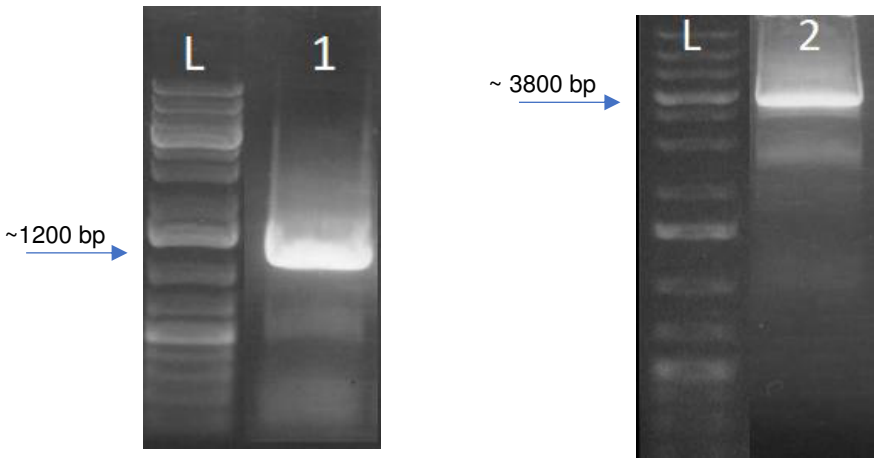

Figure 22 - Eletrophoretic analysis of the overlapped JAGGER-cYFP fragments. *L- DNA molecular weight marker; 1 – continuous JAGGER-cYFP 2+3 fragment; 2 – continuous 1+2+3 insert.*

An InFusion Cloning reaction was performed for each protein, to introduce the prepared insert into vector pH7WG. The resulting products were used to transform *E. coli.* Screening for positive colonies containing pH7WG::JAGGER::cYFP plasmid was conducted by colony PCR (fig. 23) and minipreparations for DNA extraction performed in freshly grown liquid colonies from 4 positive clones chosen.

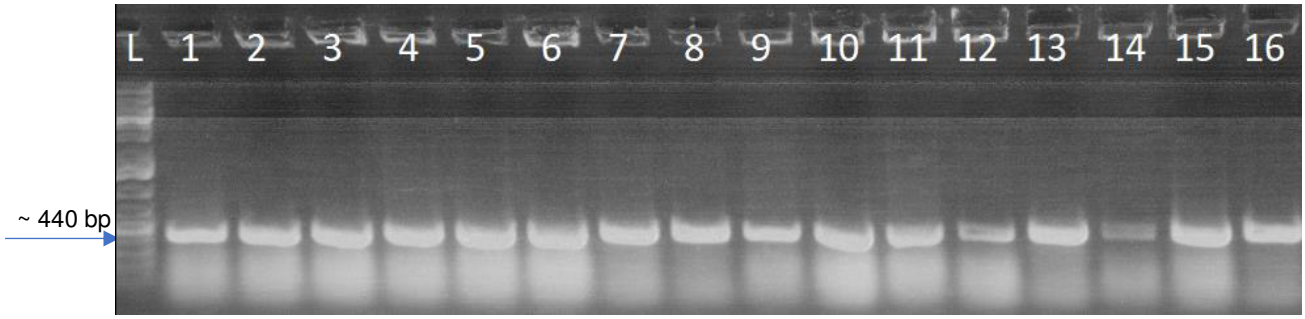

Figure 23 - Electrophoretic screening of pH7WG::JAGGER::cYFP constructs by colony PCR. *L – DNA molecular weight marker; 1-16 – citrineYFP amplified fragment signaling positive E.coli STELLAR colonies*

Restriction analyses were performed in these 4 clones, to assure correct screening, with the enzymes SpeI and AscI, and confirmed by agarose gel electrophoresis (fig 24). From the clones that presented the expected band pattern, the one with highest DNA concentration (identified in fig 24 as "1") was selected and sent for sequencing.

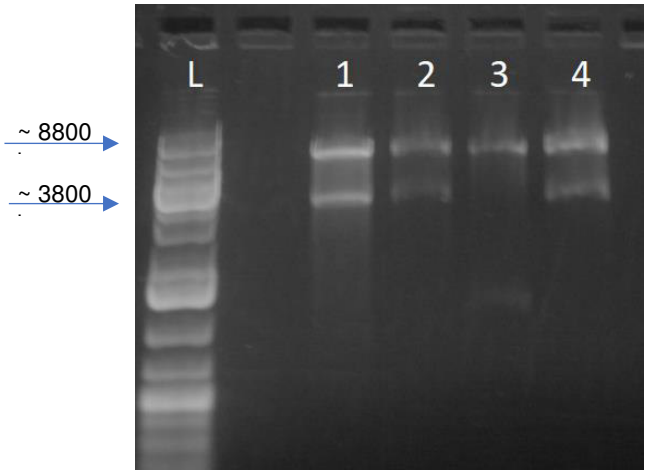

Figure 24 - Electrophoretic screening of pH7WG::JAGGER::cYFP constructs restriction analysis. *L – DNA molecular weight marker; 1,2 and 4 –positive clones; 3 – Colony PCR false positive clone.*

Transformation efficiency was noticeably higher in JAGGER (> 35 colonies) than in AGP7 (8 colonies). Screening for positive colonies containing pH7WG::AGP7::cYFP plasmid was also conducted by colony PCR (fig. 25).

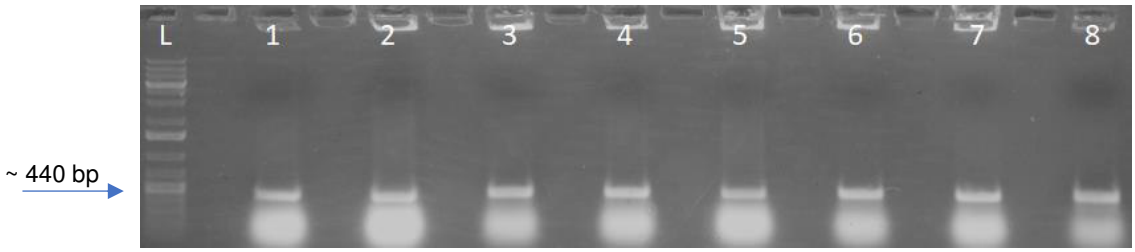

Figure 25 - Electrophoretic screening of pH7WG::AGP7::cYFP constructs after colony PCR. *L – DNA molecular weight marker; 1 to 8 – citrineYFP amplified fragment signalling positive E.coli STELLAR colonies*

DNA extracted from the positive clones obtained was subjected to restriction analysis using SpeI and AscI, however, the expected band pattern of approx. 8800 bp and 3800 bp was not observed in any of the colonies, even after repeating the restriction assay. We backtracked to the AGP7 insert, repeated the electrophoretic analysis to double check it possessed the expected size (fig. 26) and upon confirmation performed new InFusion Cloning reactions, repeated the following steps but still were unable to obtain the expected band pattern when conducting the double digestion.

To the present day we have yet to successfully achieve pH7WG::AGP7::cYFP, possibly due to insert/vector incompatibility, it will be necessary to start again with a different vector and draw new primers to complement with it.

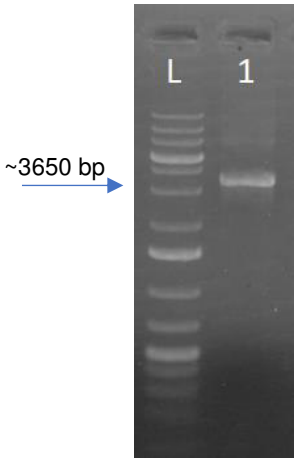

Figure 26 - Electrophoretic screening of AGP7::cYFP insert. *L – DNA molecular weight marker; 1- AGP7::cYFP PCR product*

After sequencing, the confirmed pH7WG::JAGGER::cYFP clone was cloned into *Agrobacterium* and used to transform *Arabidopsis*.

3.3.2. Obtaining the GPI signal mutants

To better understand the importance of distinct domains within the GPI signal (contained in JAGGERs nascent form) in localization and in function, we strived to achieve mutant versions in which we deleted the domain intended for study. With this in mind, we prepared the following mutants:

JAGGERΔω-site – in which we deleted the amino acid with the highest predicted probability of being the ω-site, according to the bioinformatic tool "Big PI – plant predictor" (http://mendel.imp.ac.at/gpi/plant_server.html).

JAGGERΔ2ω – in which we deleted both the amino acid with the highest and the second highest predicted probability of being the ω-site.

JAGGERΔGAS – in which we kept the predicted ω-sites, but deleted the hydrophobic tail.

Using the established JAGGER-cYFP plasmid as template, we amplified two fragments for each of the mutants (fig. 27), in order to introduce the mutation (already designed in each construct specific primers, as described in table II).

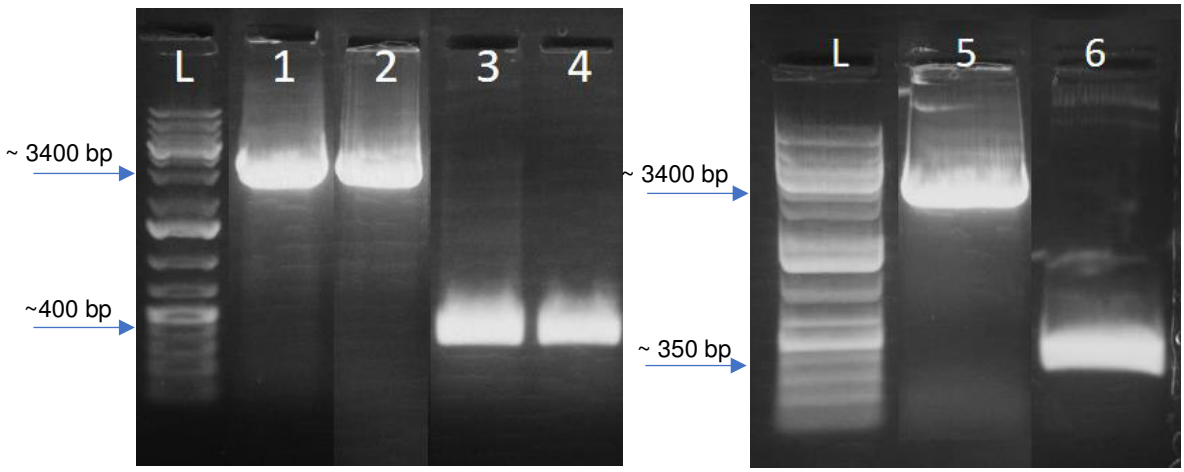

Figure 27 - Electrophoretic screening of the amplified JAGGERs mutated constructs. *L – DNA molecular weight marker; 1 – JAGGERΔωsite::cYFP fragment 1; 2 - JAGGERΔ2ω::cYFP fragment 1; 3 - JAGGERΔωsite::cYFP fragment 2; 4 - JAGGERΔ2ω::cYFP fragment 2; 5 - JAGGERΔGAS::cYFP fragment 1; 6 - JAGGERΔGAS::cYFP fragment 2*

Each mutant construct pair of fragments were made into a continuous insert, by overlap PCR, using the primers described in table I. The resulting PCR products were analysed by agarose gel electrophoresis, depicted in fig 28.

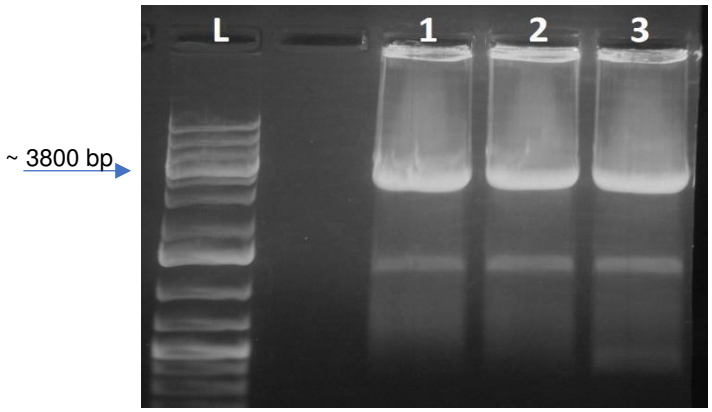

Figure 28 - Electrophoretic screening of overlapped JAGGER mutated constructs. *L – DNA molecular weight marker; 1 – JAGGERΔωsite::cYFP fragment 1 +2 ; 2 - JAGGERΔ2ω::cYFP fragment 1+2; 3 - JAGGERΔGAS::cYFP fragment 1+2*

The insert for each mutated construct was inserted into vector pH7WG by InFusion Cloning and the resulting products used to transform *E. coli.* Screening for positive colonies containing was conducted by colony PCR (fig. 29), and positive clones from each construct were selected for further screening by restriction analysis (fig.30).

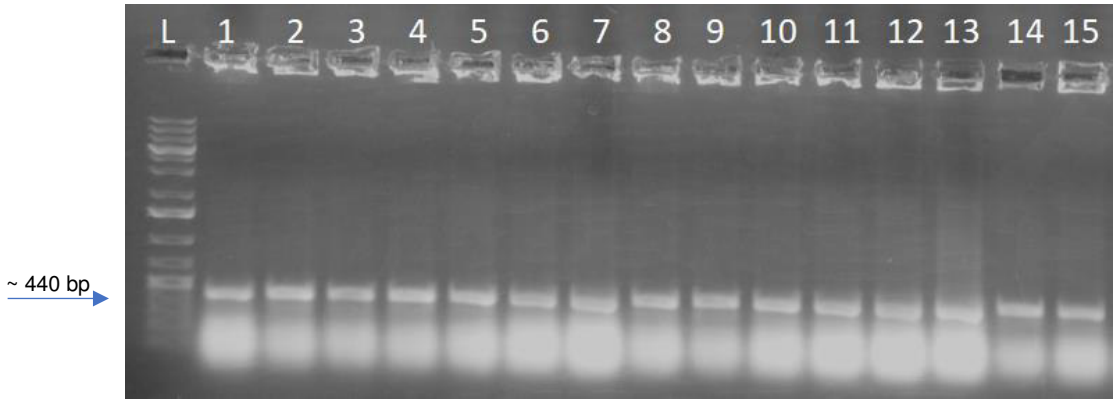

Figure 29 - Electrophoretic screening of JAGGER mutated constructs by colony PCR. *L – DNA molecular weight marker; 1 to 5 – JAGGERΔωsite; 6 to 10 - JAGGERΔ2ω; 11 to 15 - JAGGERΔGAS.*

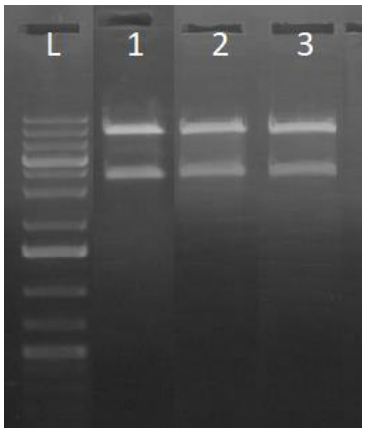

Figure 30 - Eletrophoretic screening of JAGGER mutated constructs by restriction analysis. *L – DNA molecular weight marker; 1 – JAGGERΔωsite; 2 - JAGGERΔ2ω; 3 - JAGGERΔGAS.*

One positive clone for each of the mutated constructs was sent for sequencing, and upon confirmation, transformed into *Agrobacterium*.

Transient expression in Nicotiana benthamiana

In an attempt to validate the obtained constructs functionality, we performed a transient assay in *Nicotiana benthamiana*. Figure 31 depicts the control assays: observation of uninfiltrated tissue from a plant where no leaves where infiltrated (left), and uninfiltrated tissue from a plant where one leaf had been infiltrated (right). The observation of fluorescent signal on the right, but not on the left, could indicate that the infiltrated protein was translocated through the phloem.

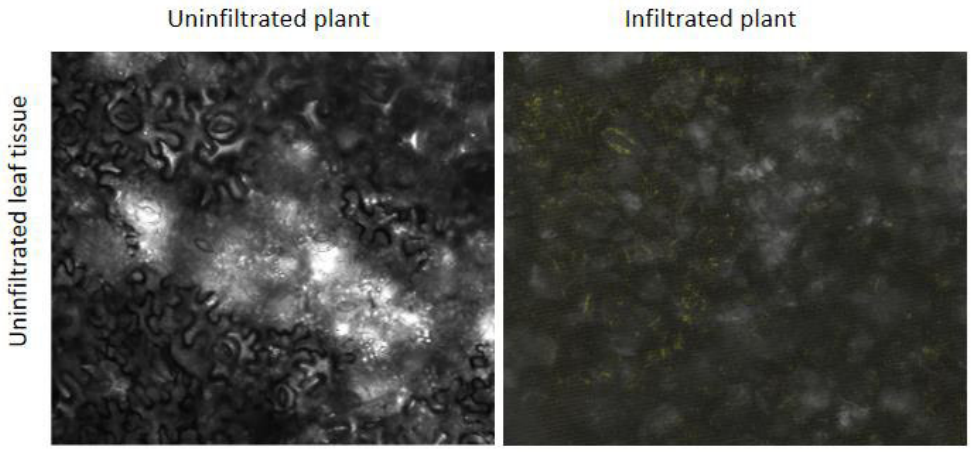

Figure 31 - Confocal microscopy observation of *N. benthamiana* uninfiltrated leaf tissue. Sample from uninfiltrated plants shows no YFP signal, whereas sample from infiltrated plant shows a diffuse YFP signal throughout the tissue**.**

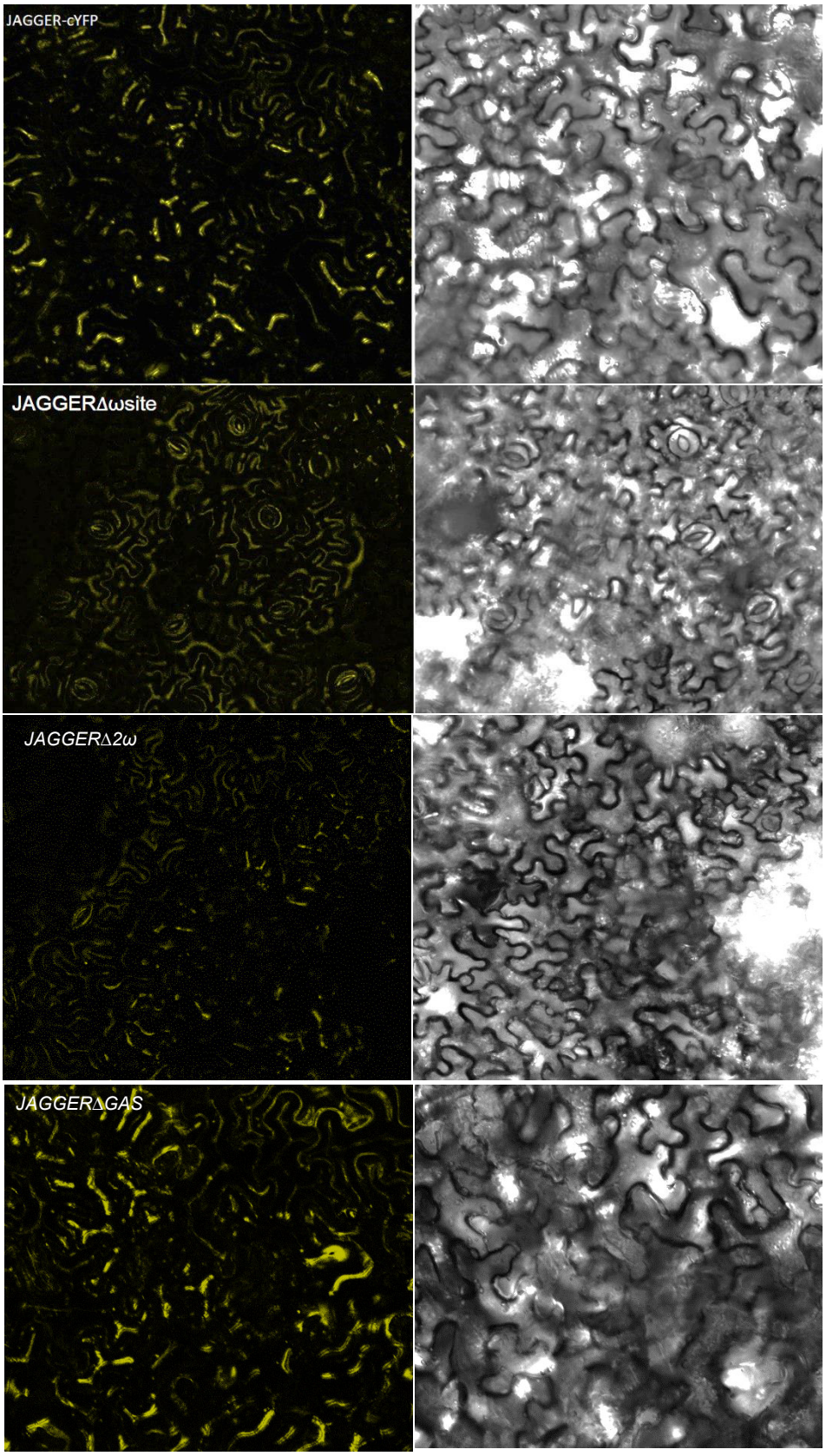

Figure 32 - Confocal microscopy observation of *N. benthamiana* leaf tissue infiltrated with JAGGER constructs. First column shows detection of the fluorescent signal; second column shows differential interference contrast (DIC)

Figure 32 depicts the observation of each experimental assay infiltrated leaf tissue: In each case we seem to detect a fluorescent signal accumulation on the inside of the puzzle-like cells, regardless of GPI signal deletions, but which seems stronger, more targeted in JAGGER-cYFP and JAGGERΔGAS, while in both w-site deletions the putative signal appears to be diffused and less intense.

As the constructs prepared are driven by their native promoters, expression will only be achieved in cells where the protein is native. According to eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) data, JAGGER is expressed in *Arabidopsis* leaves, but the database for *Nicotiana benthamiana* (https://solgenomics.net/) still possesses only an incomplete draft genome, so we could not confirm if this protein is expressed in *N. benthamiana* leaves, which renders us unable to properly evaluate these assays results.

Stable expression in Arabidopsis thaliana

Once the fusion proteins are achieved, confirmed by sequencing, and cloned into *A. tumefaciens*, we can move on to the next goal of this work: obtaining stable *Arabidopsis* lines, from various backgrounds, expressing each of the constructs.

These lines are intended to accomplish two main purposes: if (and to what extent) the JAGGER constructs rescue *jagger* plants known phenotype, and to analyze the constructs localization both in different pistil tissues and subcellularly.

Arabidopsis plants were transformed using the adapted floral dip method described in the Material and Methods, and according to the scheme depicted in figure 33.

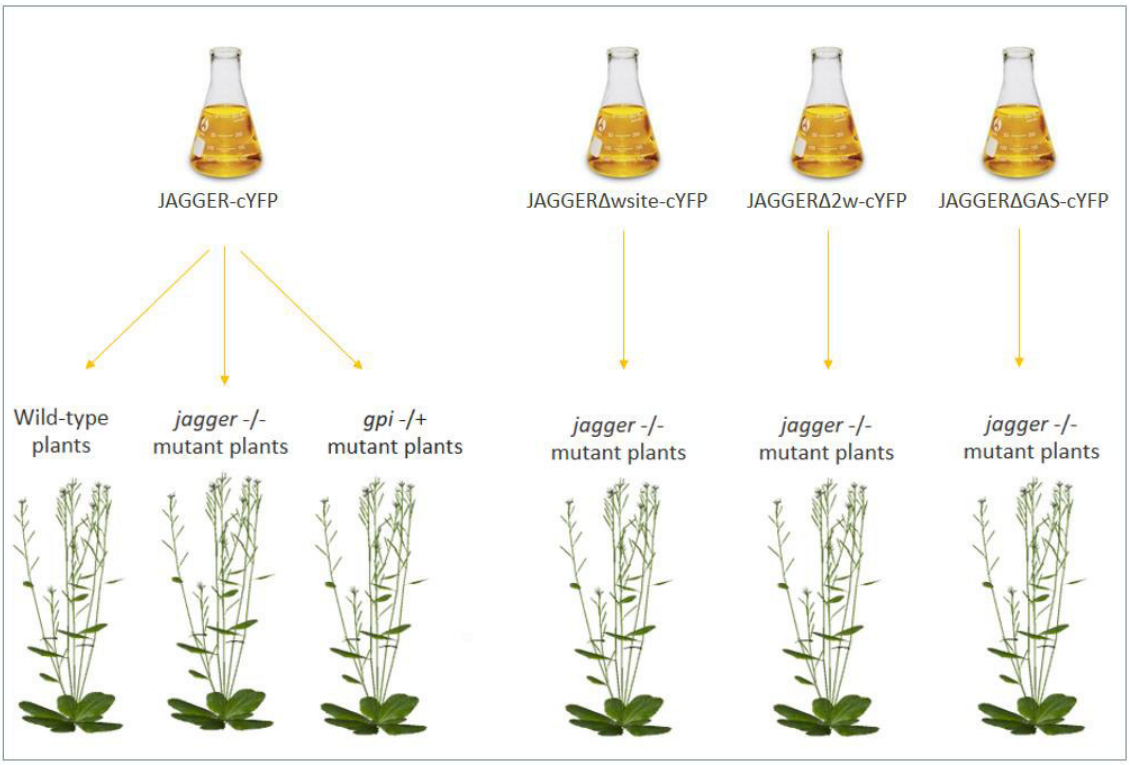

Figure 33 - Schematic representation of the constructs transformed into each *A. thaliana* background. *a) JAGGER-cYFP into Wt, jagger -/- and gpi -/+ plants; b) JAGGERΔωsite-cYFP, JAGGERΔ2 ω -cYFP c) JAGGERΔGAS-cYFP into jagger -/- plants.*

JAGGER-cYFP was transformed into:

- wild-type plants, with the purpose of observing the fusion protein localization and behavior in its natural, unaltered plant system.

- *jagger -/-* plants, with the purpose of complementing the mutant phenotype, and observing tissue and subcellular localization without native protein "interference".

- *gpi 8 -/+* plants, a mutant defective in GPI8 [Bundy *et al.,* 2016], a critical subunit of the transamidase complex which catalyzes the endoproteolysis reaction that cleaves the proteins GPI signal, before covalently attaching the GPI anchor to the ω-site. Thus, the ability for GPI anchor addition in these plants is severely impaired.

JAGGERΔωsite, JAGGERΔ2ω and JAGGERΔGAS were transformed into *jagger -/-* plants, with two clear purposes:

- Verifying their ability to complement the mutant phenotype; score the extent of the phenotype rescue in each case, and compare it to the rescue levels obtained in *jagger -/-* plants transformed with JAGGER-cYFP. This will allow us to correlate the respective GPI domain with the proteins known function.

- Observing tissue and subcellular localization; compare it to the accumulation pattern observed in plants transformed with JAGGER-cYFP in order to demonstrate the respective domains importance to the protein localization.

Selection and growth of transformants is currently underway.

3.3.3. *jagger -/-* expressing JAGGER-cYFP

Total seed yielded after transformation was collected, and screened for transformants in selective media plates. A preliminary assessment of these transformants was conducted as soon as they reached the flowering stage. Figure 34 offers a comparison of images representative of these preliminary observations (A, B), with previous published assays, studying JAGGER expression.

In A, we can observe the autofluorescence level in wild type ovules. In B, JAGGER-cYFP expression appears to be concentrating prominently in the chalazal pole of the embryo sac, and in the ovule funiculus. The *in situ* assay (C), on the other hand, highlights expression notably on the integument area, near the micropyle, which is also the case with the promoter expression GUS assay analyzing JAGGER promoter (D-G),

both in situ and GUS assays where performed by Pereira et.al, 2016, and are here reproduced with permission.

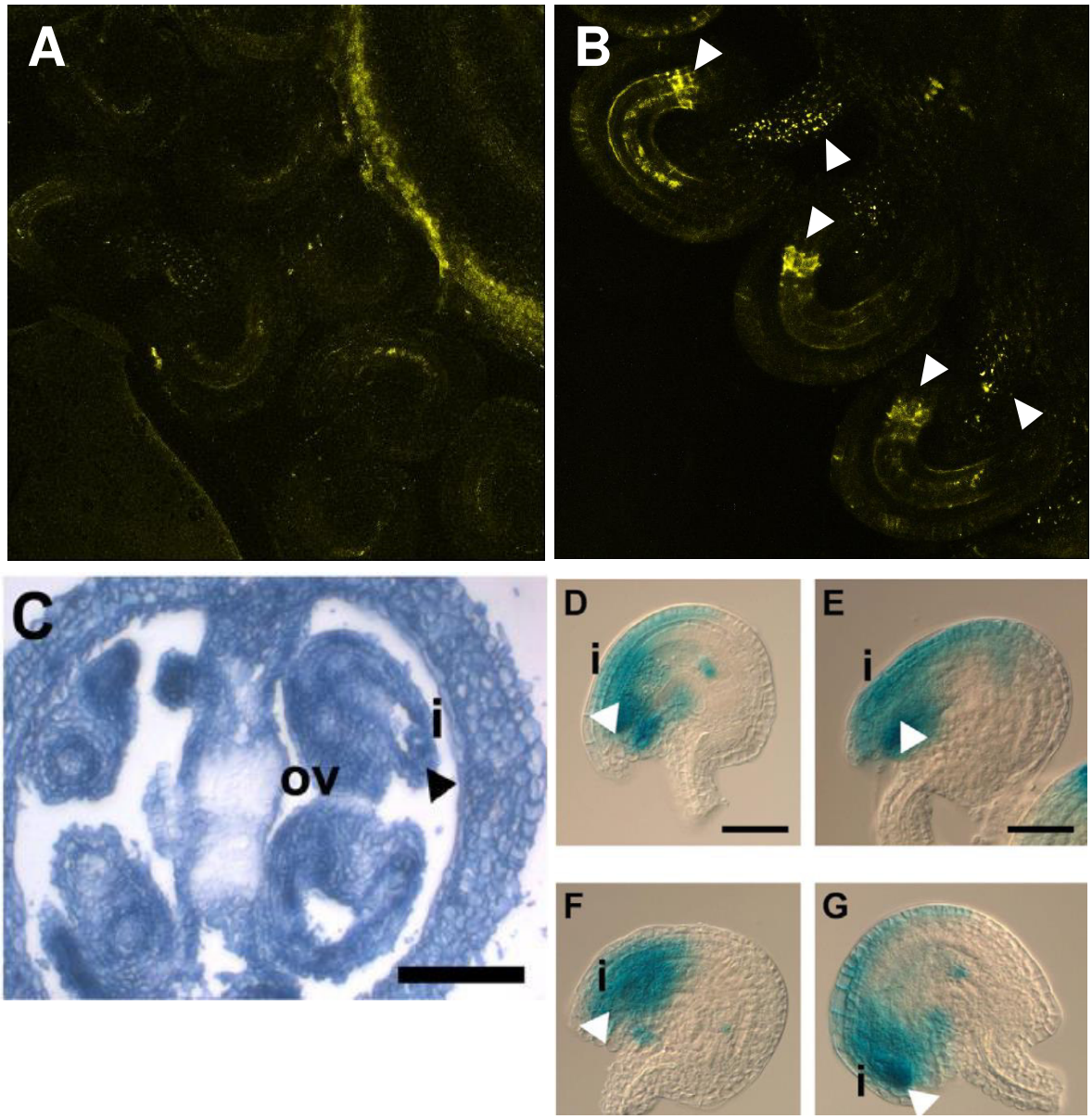

Figure 34 - Comparison of JAGGER expression in *A. thaliana* ovules.
A – Confocal observation of wild-type ovules;
B – Confocal observation of jagger -/- ovules transformed with JAGGER-cYFP;
C – In Situ Hybridization demonstrating JAGGER expression: A transverse section of wild-type pistil showing JAGGER expression in the ovule integuments and in the egg apparatus. Arrowhead indicates micropyle. (reproduced from Pereira et. al, 2016)
D,E,F,G - GUS activity driven by JAGGER promoter in the integuments near the micropyle and inside the embryo sac in the egg apparatus (arrowheads). (reproduced from Pereira et. al, 2016)

4. Discussion

The arabinogalactan protein AGP4, named JAGGER, is known to be involved in pollen tube attraction by playing a central role in the persistent synergid degeneration, however, the results obtained in previous studies have hinted that another signalling molecule, yet undiscovered, is involved in the same signalling role [Pereira *et al.*, 2016]. Phylogenetic analysis has pointed out that JAGGER and AGP7 are closely related, which could suggest an overlap in function.

LORELEI is a GPI-anchored protein that plays a critical role in double fertilization, being necessary for pollen tube growth arrest upon arrival to the receptive synergid. It has been demonstrated that, while the presence of the GPI anchor in LORELEI is determinant for its localization, it does not significantly impair its function [Liu *et al.*, 2016].

Based on this knowledge, the following working hypothesis was postulated: AGP7 plays a redundant role with JAGGER in pollen tube attraction, and both depend on its subcellular localization, determined by a GPI anchor, to effect these roles. Which raised three main questions that we tried to answer in the present work:

1. *If AGP7 has a redundant role with JAGGER in pollen tube attraction.*

To answer this question, we conducted phenotypic analysis of *agp7* null mutants. Seed set analysis results show no reduction in viable seed yielded. This is also the case for *jagger,* reported in Pereira *et al.* (2016) to yield normal seed amounts, despite the known phenotype having an indirect impact in reproductive success (polytubey causes polyspermy, which might lead to abortion of the seed). In both cases, this may be due to other players being sufficiently involved in the process to, somehow, “pick up the slack” as we can see in chapter 1. figure 10, JAGGER and AGP7 belong to a closely related group of 5 proteins (AGP2, AGP4, AGP7, AGP5 and AGP1), and it might need a mutant that knocks out the 5 of them to achieve an observable failure in reproduction output. None of these proteins, other than JAGGER, have been studied regarding their roles, so closely analysing them is likely the next step to pursue in this research.

Polytubey occurrence was also a main concern: we looked particularly into this step due to the known *jagger* phenotype having polytubey increase (but not full penetrance, as stated in Pereira *et al.* (2016)) and so we wanted to check if the partial complementation was given by the presence of AGP7, in which case we would expect a similar rise in polytubey occurrence in *agp7* -/- mutants. The aniline blue assay results obtained so far seem to indicate that this is not the case.

We know that AGPs work as signalling molecules [Pereira *et al.*, 2016] and that JAGGER in particular, acts in the signalling pathway that carries the notice of double fertilization success to the persistent synergid, thus inducing its degeneration. Although this is the only currently known JAGGER role, it is certainly not its only role, given the variety of tissues it is expressed in. It might be the case that AGP7 works together, or in succession, with JAGGER in another, yet to be uncovered role.

GUS promoter assays for both *JAGGER* [Pereira *et al.*, 2016] and *AGP7* [Pinto *et al.*, unpublished data] demonstrate that regarding pistils tissues, both proteins are sometimes present on the same tissue (overlapping), or in adjacent tissues in a sequential distribution (complementary). In table XI the observed promoter expression is summarized, for convenience. This points to the possible contribution of both proteins to converging processes, and strengthens the need to study *AGP7* separately, in all of these tissues.

Table XI - Summary of *JAGGER* and *AGP7* promoter expression in *Arabidopsis* pistils

	JAGGER	*AGP7*	Complements	Overlaps
Stigma	+	-	Yes	----
Style	+	+	----	Yes
Transmitting tract	+	-	Yes	----
Funiculus	-	+	Yes	----
Integument	+	+	----	Yes
Egg apparatus	+	+	----	Yes

With this in mind, we also worked on obtaining a double homozygous *jagger agp7* mutant in order to analyse its phenotype in comparison to both single mutants. We expect that

in this mutant the occurrence of the polytubey phenomenon increases when compared to *jagger,* and it will be interesting to see what differences we might find in this double mutant regarding, for example, pollen tube growth and guidance in the pistil.

2. *If the predicted GPI anchor determines the protein subcellular localization.*

To answer this question, we first needed to establish the proteins default subcellular localization. In order to achieve this, we prepared fusion proteins between each protein of interest and the reporter gene cYFP. We chose the "citrine" version of YFP reported [Griesbeck *et al*., 2001] as pH insensitive, that would not be impaired by the acidic environment of the apoplast [Gjetting *et al*., 2012], where we predict these proteins to accumulate.

Due to the complications already addressed in chapter 3 "Results", AGP7s fusion protein was unfortunately not achieved in the duration of this work.

Nascent JAGGER and its GPI signal-mutated versions were used to transform *Arabidopsis*, stable lines are still being established and so we have yet to achieve conclusive results regarding protein localization.

When analysing the transformed proteins, being chimeric constructs, we must take the following into consideration:

Regarding JAGGER-cYFP, both proteins are linked by a stretch of 9 amino acids, adapted from the one used in LORELEI-cYFP [Liu *et al*., 2016]. This linker was used in all of the constructs obtained in the course of this work, and is composed of the following sequence:

Gly - Arg - Pro - Gly - Gly - Gly- Gly - Gly - Ala

Gly^9 is a common flexible linker [Chen *et al*., 2013], which confers easy mobility and interaction to the fused proteins. However, fully flexible linkers have been reported in some instances to have poor expression yields or loss of biological activity.

Pro and Ala are typical amino acids of rigid linkers, so this linker was designed to bring the most benefits from both and may be classified as a semi-flexible linker: which is

intended to allow us to benefit from having freedom of movement, but still keeping an effective separation of the two proteins and prevent them from interfering with each other.

Flanking cYFP, a repeated JAGGER sequence of 11 amino acids was placed, a proline and alanine rich, unstructured domain entitled ω-11. This domain is typical in GPI-anchored proteins and may be considered a GPI signal companion [Liu *et al.*, 2016]. We chose to structure the construct in this manner for the following reasons: Cleaving the AGP for fusion in this domain keeps the coding sequence unaltered, and so should not impair protein folding nor function; seeing as this domain is constantly found next to the ω-site, it might be necessary for the proper processing of the protein by the transamidase complex, perhaps as a regulatory sequence or enhancer. To prevent hindering the GPI anchoring mechanism should that be the case, we placed a repeated ω-11 domain between cYFP terminus and ω-site, which should solve any need for regulatory cues, keeping the same amino acid sequence but altering the codons used (observing *Arabidopsis* codon bias) which should prevent over enhancement. Figure 35 depicts the positioning of said domains in the construct.

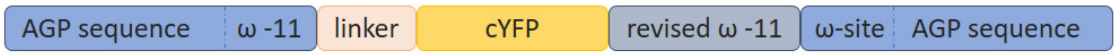

Figure 35 - Schematic representation of the positioning of ω -11 regions included in all constructs obtained. Not drawn to scale.

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Transient expression in Nicotiana benthamiana

In an attempt to test the constructs functionality, we set up a transient assay in *N. benthamiana* leaves, in parallel with the stable transformations. This model was chosen out of convenience and time effectiveness, since it was already being used in Dr. Palanivelus' lab and grown plants readily available. However, due to *N. benthamiana* available genome being still a draft, we were unable to ascertain the expression of JAGGER in its leaves.

If it is indeed expressed from its native promoter in a mesophyll cell, we would expect the protein to follow the secretory pathway (due to the signal peptide presence, and absence of other sorting determinants) until arriving on the plasma membrane, upon which it should remain anchored to the membrane (due to possessing a GPI anchor).

Comparing the signal accumulation patterns obtained (fig. 32) to the pattern of a plasma membrane protein [Kanehara *et al.*, 2015], which shows an accumulation pattern of

continuous fluorescence around the "puzzle piece"-shaped cells, we see that none of the infiltrated constructs shows a similar pattern - on the contrary, we seem to only detect a fluorescent signal in the cells interior. We might consider that the protein might be arrested in the ER due to problems in its transaction or folding, but it does not correspond to the mesh-like pattern [Boevink *et al.*, 1996] of fluorescent ER retained proteins. We might also consider that the protein, not having passed the cells quality test, is being directed to the vacuole, which composes the majority of the cells interior.

However, upon comparison with the negative controls we observe that un-infiltrated leaf tissue (fig. 31), collected from a different leaf but from a plant that has been infiltrated on other leaves, shows the same signal accumulation pattern. This might happen because of systemic transportation or simply because it is the basal, auto-fluorescence of these tissues. We have to conclude we possess insufficient knowledge of this proteins expression in the plant, and of the system itself, to be able to obtain good data yielding useful information, so we deem this assay's results inconclusive.

Stable expression in Arabidopsis thaliana

From the GUS and *in situ* expression assays presented in Pereira *et al*. (2016), we expect JAGGER-cYFPs' accumulation pattern to be stronger in the integument surrounding the micropylar area and in the synergid cell, regarding the ovular tissues, and surrounding the cell, in the plasma membrane, with particular emphasis in the filiform apparatus, regarding the subcelular localization in the synergids.

The preliminary images shown in Figure 33, seem to demonstrate however an accumulation pattern in the opposite pole of the ovule, the chalaza, and there is also an intriguing stronger signal in the funiculus, which is not present neither in GUS nor *in situ*. The size of the ovules in this figure lead us to think they might already be fertilized, which could explain the unexpected signal accumulation: synergid cells are only present before fertilization and the expression patterns post fertilization might be altered, but in that case we would expect the accumulation pattern to be diffused along the developing seeds' interior, seeing as the synergids degenerate and are fused with the expanding endosperm. It might be the case that we need an F2 generation established before we properly analyse the constructs expression, as was the protocol followed in Liu *et al*. (2016). Further observation is needed before we are able to draw conclusions.

We also prepared mutated versions of JAGGER-cYFP in which GPI signal domains were modified, in order to correlate each domain with the protein localization.

In the ω-site deletion, we removed the amino acid predicted *in silico* to be the GPI anchor attachment site (fig. 8). This reasoning led us in fact, to prepare two constructs, due to the existence of two possible ω-site predictions. In Liu *et al*. (2016), the GPI-anchored protein LORELEI was shown to accumulate almost exclusively in the filiform apparatus in the synergid cell, and no observable difference arose from loss of the single amino acid predicted as ω-site. The authors proposed this was due to a second, cryptic, ω-site being still present and ran a second prediction after deletion: finding a new predicted ω-site. Upon loss of these two predicted amino acids filiform apparatus localization diminished dramatically. We applied the same reasoning and prepared one construct with the single, first predicted ω-site deletion (JAGGERΔωsite), and another construct in which we deleted both the first and the second predicted ω-sites (JAGGERΔ2ω). We expect to verify some level of altered signal accumulation in JAGGERΔ2ω, if not in both of the constructs, in fact, the second predicted ω-site in JAGGERs sequence has a much lower confidence value associated than LORELEI – which would be consistent with a likely disruption of the GPI anchor addition.

We also prepared a mutated construct which deleted the entire hydrophobic region in the GPI signal, the GAS domain, leaving the ω-site intact. Mao *et al*. (2003) observed a different pattern in accumulation of a GPI-anchored protein upon loss of its GAS domain, contrary to what happens with LORELEIs ω-site deletions, that diminishes the signal accumulation in the FA while increasing the signal in the synergid cytoplasm, GAS deletion resulted in the protein accumulating in the extracellular space, with a visible increase overtime. This suggests that, keeping the ω-site but losing the hydrophobic domain guarantees the protein correct sorting, but incapacitates its anchoring in the plasma membrane.

3. *If AGP7/JAGGER functions depend on the protein subcellular localization.*

This step is dependent on achieving step 1 (uncover an *agp7* mutant phenotype in reproduction) and step 2 (obtaining the described fusion proteins and determining their subcellular accumulation patterns) successfully. To tackle this question, we would

express the protein-reporter fusions, in *jagger* and *agp7* single mutant plants, to determine if there is a phenotype rescue (mutant plant reverts to wild type parameters of the observed phenotype) to some degree. This will tell us if the protein is functional and validate both function and localization studies.

We will not be conducting this study in *agp7* single mutants, since we have neither identified a phenotype so far, nor achieved the AGP7 – cYFP fusion protein.

In regard to JAGGER, if upon transforming JAGGER-cYFP in jagger -/- plants we observe that there is less polytubey occurrence, we will consider the fusion protein functional and proceed to analysing the mutated versions.

To correlate the protein localization with its function, we must select the mutated constructs that have shown differential expression pattern when compared to nascent JAGGER, and transform them into *jagger* -/- plants. If the polytubey occurrence in these plants diminishes to a noticeable extent, then the change in localization did not have a dramatic effect in the proteins ability to fulfil its role. In the LORELEI study, Liu *et al*. (2016) have observed precisely this: *lorelei* -/- phenotype (pollen tube coiling inside the synergid due to not arresting its growth), although never reverting to the same level as wild type, still diminished considerably in its occurrence, demonstrating that localization has an impact, but is not essential for LORELEI function.

Future Perspectives

In the course of this work we have developed tools that will be of value in future studies, helping to shed light into the GPI anchor mechanism in respect to protein dynamics, and improving our knowledge of AGPs in sexual reproduction.

The following steps are, first and foremost, to analyse through confocal microscopy the stable *Arabidopsis* lines which are being screened for each JAGGER construct, and to observe the polytubey occurrence in these lines (on going work). Also to analyse the double homozygous mutant being obtained.

Future studies would include:

- Thorough observation of *agp7* plants reproductive tissues;

- To achieve an AGP7-cYFP fusion protein and analyse it accordingly;

- To perform promoter swaps constructs between JAGGER and AGP7 and transform them in the corresponding mutants;

- To study the other closely related AGPs and obtain a five knock-out mutant.

We believe the present study and resulting work will be useful in laying essential foundations on which biotechnology can build upon for the future improvement of seed production.

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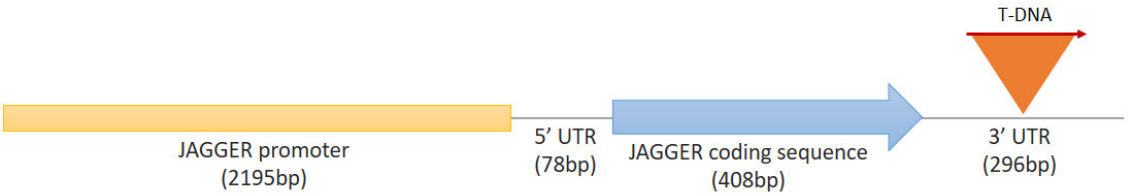
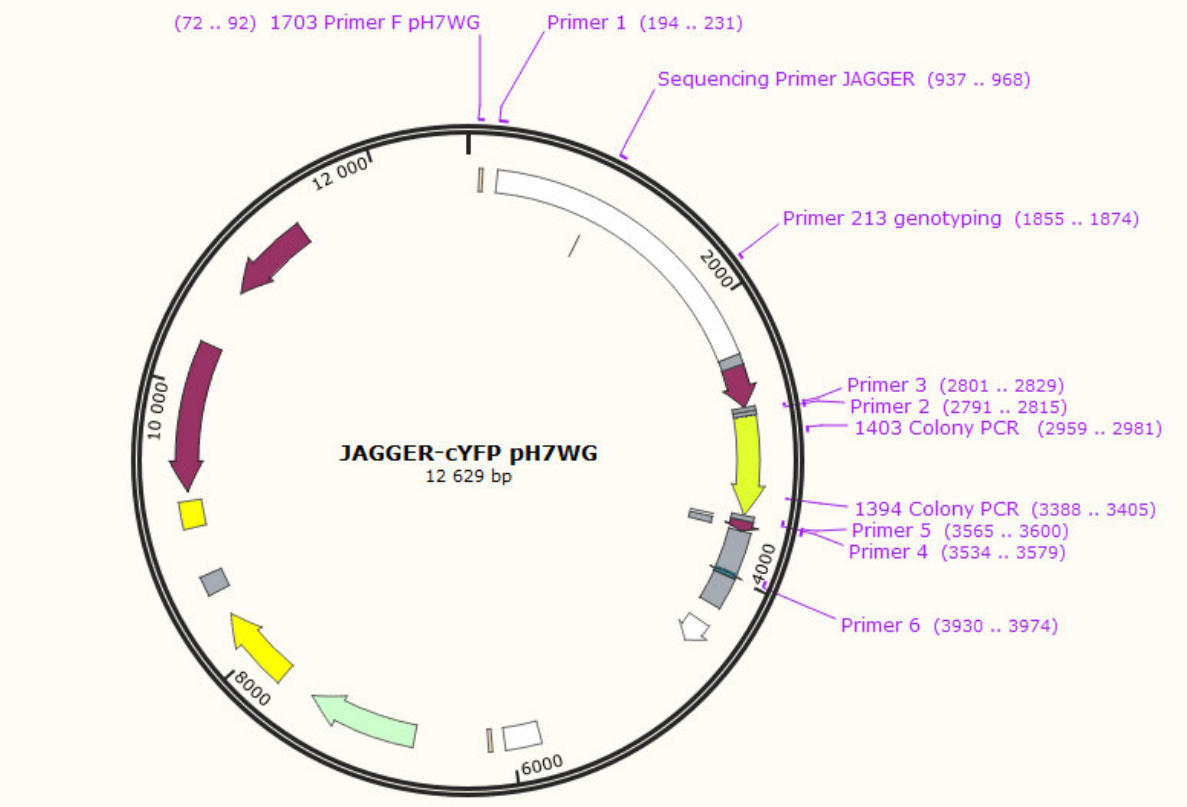
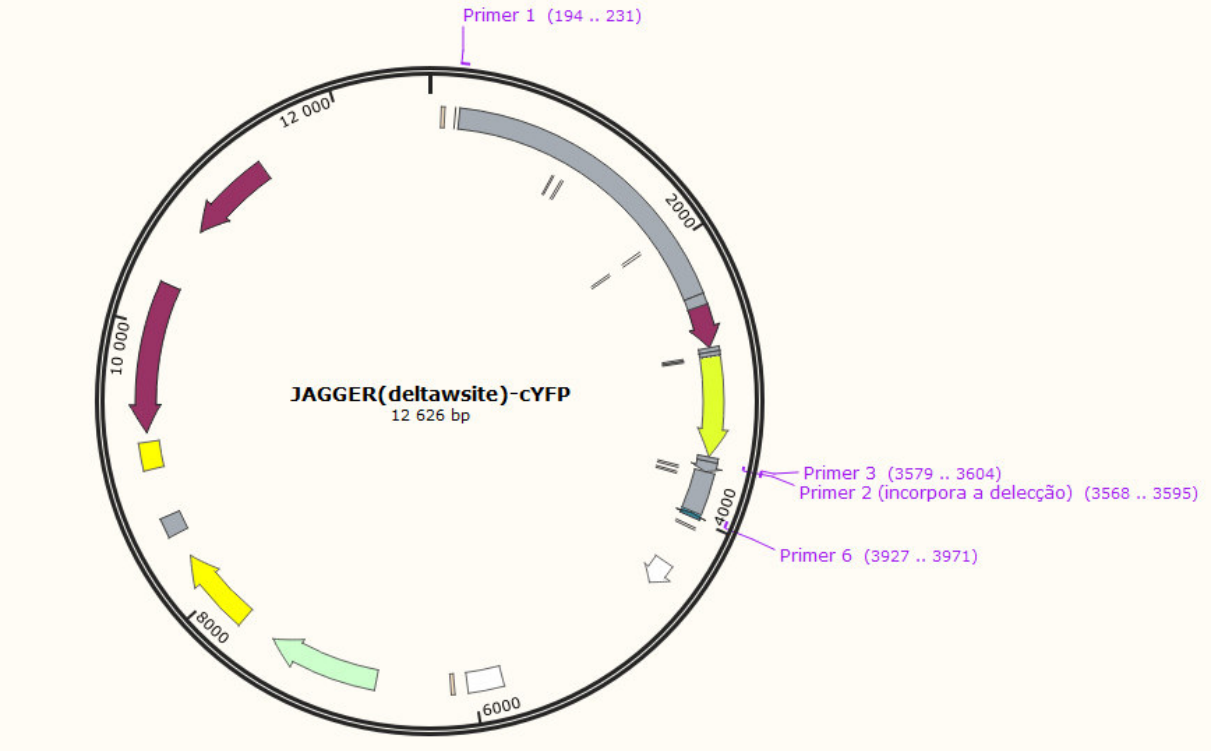
Supplemental Data



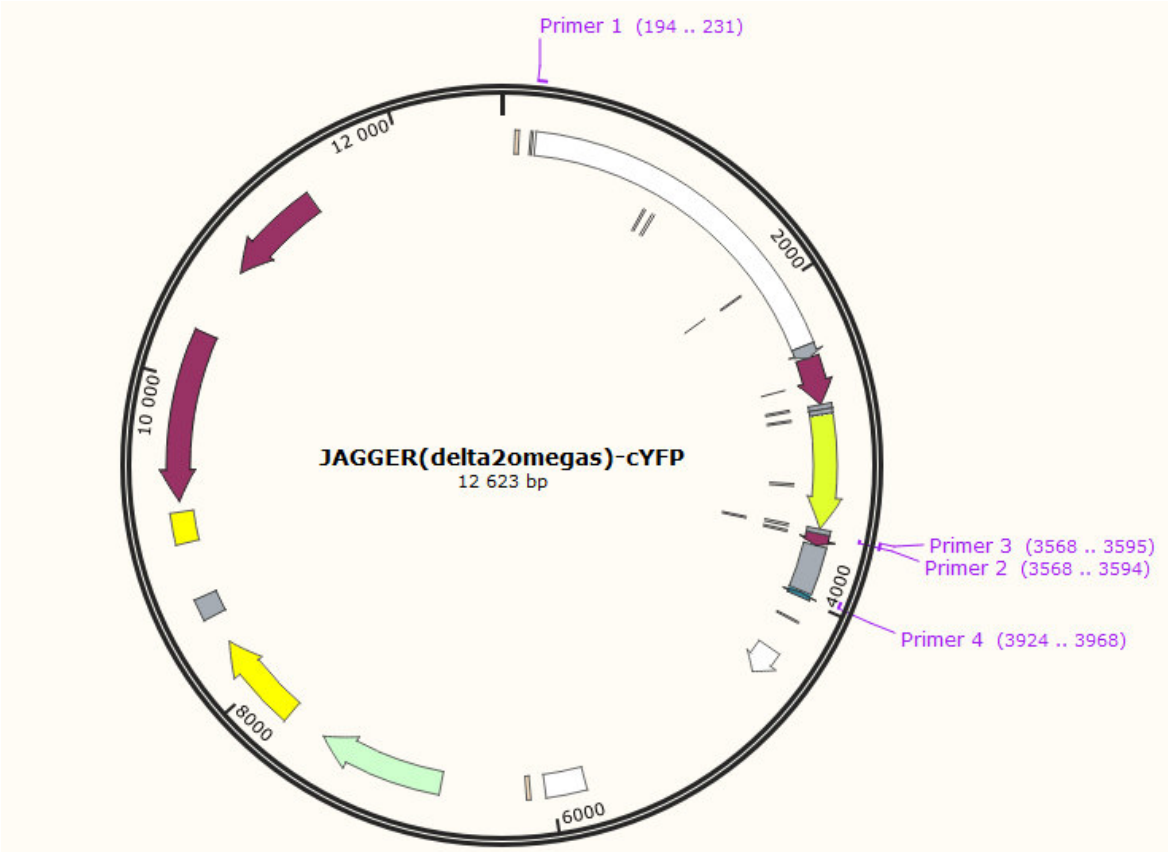
Supplemental image 1: Schematic representation of the JAGGER gene and the T-DNA insertion site for the mutant allele used in this work



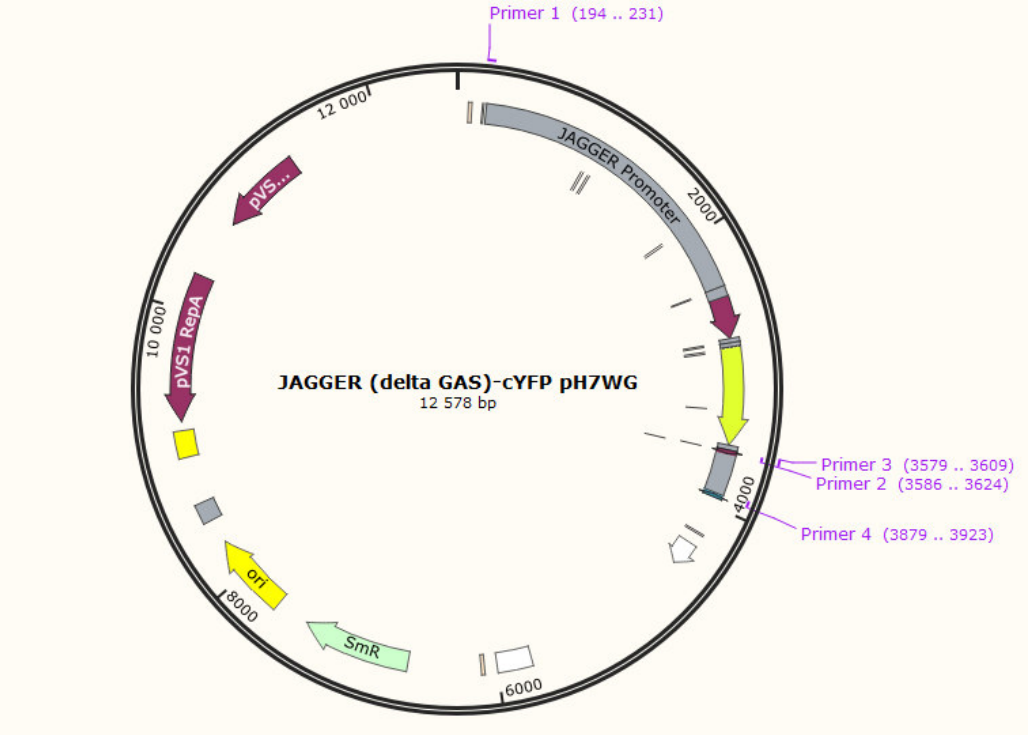
Supplemental image 2: JAGGER – cYFP plasmid map (SnapGene)



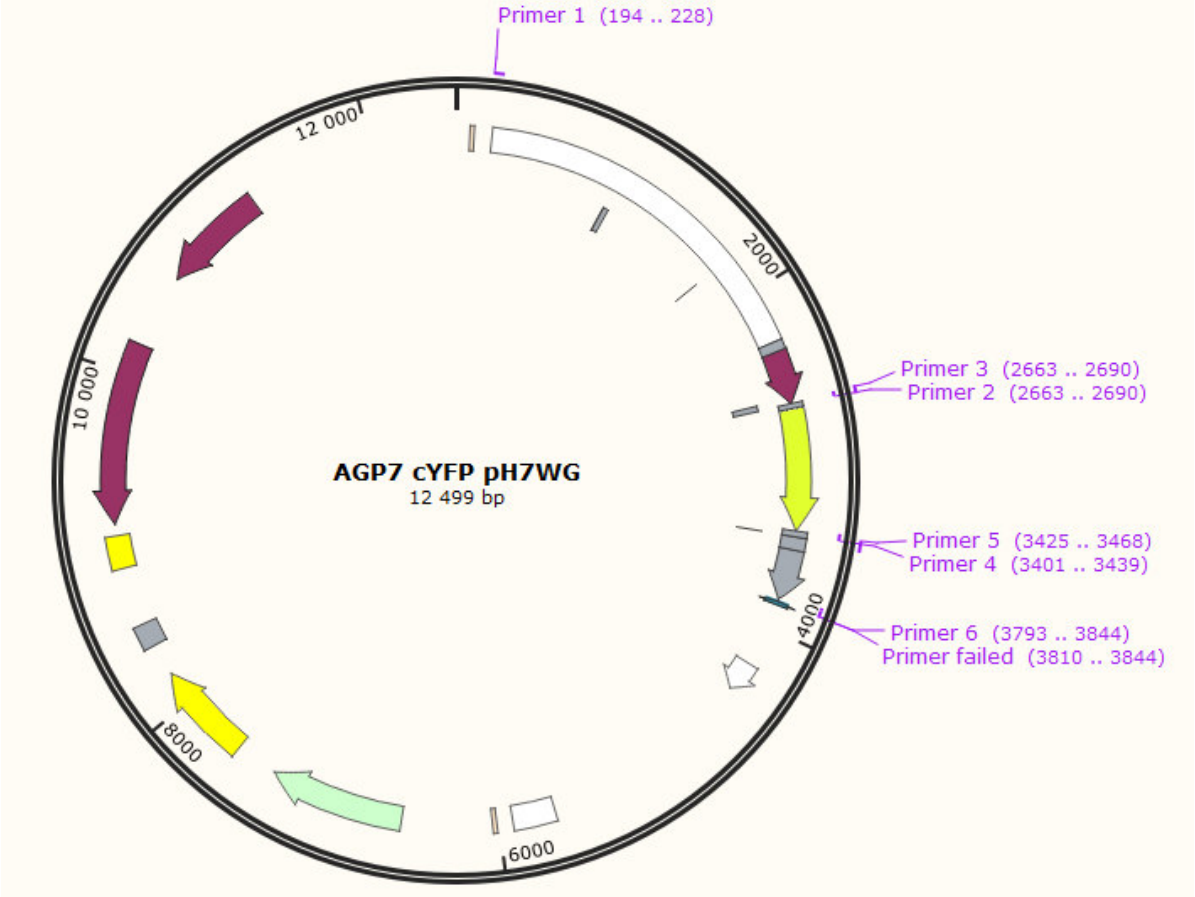
Supplemental image 3: JAGGERΔωsite – cYFP plasmid map (SnapGene)



Supplemental image 4: JAGGER∆2ω – cYFP plasmid map (SnapGene)



Supplemental image 5: JAGGERΔGAS – cYFP plasmid map (SnapGene)



Supplemental image 6: AGP7 – cYFP plasmid map (SnapGene)